ACCSCIENCE PUBLISHING

Tumor Discovery

ORIGINAL RESEARCH ARTICLE

Effect of 900 MHz radiofrequency electromagnetic radiation emitted from mobile phone on testicular immunity and the associated risk of testicular germ cell tumor

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Abstract

The emission of radiofrequency electromagnetic radiation (RF-EMR) from mobile phones has been implicated in causing inflammatory changes in the testis. Nevertheless, the direct association of these changes with the development of testicular germ cell tumors (TGCT) remains unclear. Therefore, we purposed to investigate the effect of RF-EMR exposure on inflammatory changes in the testis, cytokine gene expression levels, and the incidence of TGCT. Twenty male Wistar albino rats were randomly assigned to either the study or control groups. The study group was exposed to RF-EMR at 900 MHz, 26 V/m, and a specific absorption rate (SAR) of 0.14 W/kg for 4 h/day over 8 weeks. Histopathological analysis, vitality analysis using Annexin V, and real-time polymerase chain reaction for the analysis of interleukin 1 (IL-1), IL-4, IL-10, tumor necrosis factor-alpha, and interferon-gamma (IFN-γ) cytokine gene expressions were performed on the testicular tissue. The median testis weight (163.0 g [133.0 – 183.0] vs. 179.0 g [134.0 – 195.0], $P = 0.012$) and volume (0.95 cm^3 [0.800 – 1.400] vs. 1.100 cm^3 [1.050 – 1.500], $P = 0.031$) of the study group were significantly lower compared to the control group. The seminiferous tubule damage ($P < 0.001$) and interstitial edema ($P = 0.042$) were significantly higher in the study group. The tunica albuginea thickness was significantly reduced in the study group ($P < 0.001$). The fold changes in the expression levels of IL-4 and IFN-γ increased significantly in the study group. Our findings indicate that RF-EMR exposure causes structural, histopathological, and inflammatory toxic effects on the testis. The observed elevation in gene expression levels of IL-4 and IFN-γ cytokines following RF-EMR exposure suggests their potential role as regulators of TGCT initiation, thereby offering a viable potential therapeutic target in combination with current treatments. Nonetheless, future well-designed studies are necessary to validate our findings.

Keywords: Radiofrequency electromagnetic radiation; Testis; Inflammation; Testicular germ cell tumor

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1. Introduction

Due to its advanced technological features, the prevalence of mobile phones has increased considerably. Investigators have suggested that the radiofrequency electromagnetic radiation (RF-EMR) emitted by mobile phones poses deleterious effects on human health.[1] Studies have demonstrated that RF-EMR from mobile devices may be associated with various adverse effects at molecular and cellular levels, including cancer, oxidative stress, increased free radicals, lipid peroxidation, DNA damage, and chromosomal abnormalities.[1] The testes and brain are among the organs most extensively investigated owing to their intense exposure to RF-EMR.[1] RF-EMR generally exerts negative effects on seminal parameters in the testes, such as sperm count, concentration, motility, and percentage of normal morphology. Moreover, it disrupts the morphological structure of the testes and boosts the permeability of the blood–testis barrier.[1]

Cytokines are physiologically produced in the testes and play essential roles in maintaining their normal function. However, cytokines also have critical roles in inducing inflammatory reactions under stress conditions. Exposure to RF-EMR leads to an increase in reactive oxygen species (ROS) and oxidative stress.[2] ROS generated by oxidative stress activates nuclear factor kappa B (NF-κB) and activator protein-1. In addition, it stimulates the transcription of genes encoding growth factors, cytokines, and extracellular matrix proteins.[1]

Testicular germ cell tumors (TGCT) are the most prevalent solid tumors in young men and commonly exhibit infiltration by T lymphocytes.[3] Tumor-infiltrating lymphocytes (TIL) and cytokine-mediated immunity are significantly associated with the development and prognosis of TGCT.[4] Klein *et al.* observed numerous B, T, and dendritic cells within germ cell neoplasia *in situ* (GCNIS) and seminoma.[4] Furthermore, a spectrum of cytokines, including pro-inflammatory cytokines (interleukin 1 beta [IL-1β], IL-6, and tumor necrosis factor-alpha [TNF-α]), anti-inflammatory cytokines (transforming growth factor-beta [TGF-β1), Th1-mediated cytokines (IL-2 and interferon-gamma [IFN-γ]), and chemokines (chemokine CXC ligand 13 [CXCL-13], chemokine CXC ligand 10 [CXCL-10], and chemokine [C-C motif] ligand [CCL-5]), were significantly presented in TGCT, suggesting the presence of a pro-tumorigenic milieu.[4] This experimental study aimed to investigate the impact of RF-EMR on inflammatory cytokine gene expression levels in the testes and its potential association with the development of TGCT.

2. Methods

2.1. Experimental design

The experimental study was conducted at the Cukurova University Health Sciences Experimental Application and Research Center. Male Wistar albino rats were utilized for the study. A total of 20 rats were categorized into study and control groups (10 rats each). Throughout the 8-week duration of the study, the rats were fed with adequate tap water and standard rat pellet chow without dietary restrictions imposed. All experimental procedures were conducted in accordance with the Institutional Animal Ethics Committee recommendations (Protocol Authorization Number: 5-2-2019).

A specialized application cage measuring 90 cm × 90 cm × 42 cm, consisting of four equal sections to accommodate rats comfortably during exposure to RF-EMR, was custom-designed (Figure 1A). The study used a radiofrequency electromagnetic field (RF-EMF) generator (GHZ2011X, Set Electronic Co. Ltd., Turkey). A monopole antenna connected to the generator emitted 900 MHz RF-EMR and was placed in the application cage. The monopole antenna was strategically positioned to evenly radiate RF-EMR at a consistent distance of 32 cm from the bodies of the rats in the study group (Figure 1B). In addition, temperature changes resulting from RF-EMR exposure were monitored using a four-channel thermometer. The specific absorption rate (SAR) value was computed based on the calculated electric field density (V/m) and power density (mW/cm^2). For the numerical determination of SAR, CST (Microwave Studio Suite [version 2018], Dassault Systèmes, Germany), an electromagnetic field solver utilizing the finite integration technique, was used (Figure 1C and D). SAR values ranged from 0.008 to 0.14 W/kg. Therefore, under identical conditions, orientation, and antenna power, a uniform SAR value of 0.14 W/kg across the whole body was

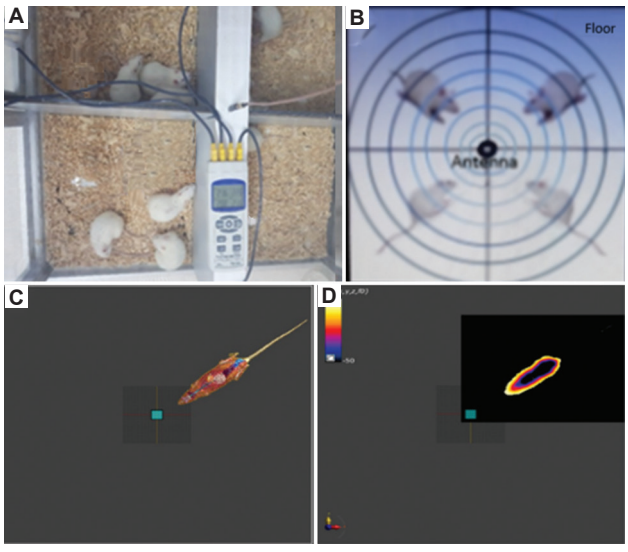

Figure 1. (A) Radiofrequency electromagnetic radiation exposure system. Monopole antenna placed in the application cage (B) and specific absorption rate distribution for the whole body in the voxel mouse model (C and D).

derived. Over the course of 8 weeks, the rats were exposed to RF-EMF at 900 MHz, 26 V/m, and SAR 0.14 W/kg for 4 h/day. At the end of the 8-week exposure period, all the rats from both the control and study groups were sacrificed, and orchiectomy procedures were performed. Testicular weight and volume were recorded, followed by storage of testis tissue for subsequent histopathological examination, DNA analysis, and apoptotic cell analysis.

2.2. Real-time polymerase chain reaction (PCR) gene expression analysis

Gene expression profiling for IL-1, IL-4, IL-10, TNF- α, and IFN-γ was conducted using primer-based designed specifically for these genes, following the protocols outlined below.

2.2.1. RNA isolation from tissue

Tissue sections with a thickness of 10 μm were collected, and the isolation protocol was performed in accordance with the manufacturer's directions (RNeasy FFPE Kit, Qiagen, Germany). Following isolation, RNA concentration was measured.

2.2.2. cDNA synthesis protocol

Ten microliters of RNA were transferred to a 0.2 ml PCR tube and incubated at 65°C for 5 min in a PCR instrument (VeritiPro Thermal Cycler, ThermoFisher Scientific, USA). A cDNA mix was prepared with 7.5 μL of RT mix and 2.5 μL of reverse transcriptase, followed by initiation of the cycle in the PCR instrument. The High-capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, USA) was used for cDNA synthesis.

2.2.3. Real-time PCR protocol

Real-time PCR experiments were performed using the GoTaq RT-PCR kit (Promega, USA) and SYBR Green chemistry (Bio-Rad, USA). The primer sequences used in this study are provided in the Appendix. Both procedures were conducted according to the manufacturer's instructions. A mixture comprising 20 μL RT-PCR mix and 5 μL cDNA was dispensed into each of the reaction tubes, which contained samples, negative control, positive control, and standards, respectively. Subsequently, the RT-PCR protocol was started in the PCR instrument. Upon completion of the RT-PCR protocol, the data obtained were analyzed using the delta cycle threshold (Ct) (comparative Ct method [ΔCt], 2-DDCt) method. Ct values for both the control and study groups were obtained from experiments performed using the qRT-PCR Rotor-Gene Q (Qiagen, Germany) device to determine changes in the expressions of IL-1, IL-4, IL-10, TNF- α, and IFN- γ genes. The changes in the expressions of the relevant genes were calculated by entering Ct data into Microsoft Excel. For statistical analyses, the Gene Globe Data Analysis Center (Qiagen, Germany) online analysis program was utilized.

2.3. Vitality determination using flow cytometry

Fresh tissue samples from both the control and study groups were cut into pieces and separated in sterile Petri dishes. They were centrifuged twice for 5 min at 400 relative centrifugal force, resulting in the suspension of cells at the end of these processes. After the final centrifugation, the cell pellet was resuspended with 100 μL of a binding solution, resulting in a 10-fold dilution, and maintained on ice. Next, 5 μL of Annexin V-FITC solution (Beckman Coulter, France) was added to the cell suspension and incubated on ice for 10 min in the dark following gentle mixing. After incubation, 400 μL of the binding solution was added, and readings were obtained using a flow cytometry device (Cytoflex, Beckman Coulter, USA). Subsequently, Annexin V-FITC-positive cells were selected, and apoptotic and necrotic cells were determined. Cells selected from the side scatter (SSC)/forward scatter (FS) graphics and analyzed in Annexin V/PI graphics.

2.4. Histopathological examination of testicular tissues

The testis resection samples intended for histopathological examination were initially fixed using 10% formaldehyde. Subsequently, five-micron-thick sections were obtained from the paraffin-embedded tissues. These tissue sections, prepared on specialized slides, were stained with hematoxylin and eosin (H&E) staining using an automated staining device (Leica ST 5020, Leica, Germany), followed by automated closure using another device (Leica ST 5030, Leica, Germany). In addition, tissue samples were subjected to histochemical analysis using Masson's trichrome method. Immunohistochemical (IHC) staining was performed on five-micron-thick sections of formalin-fixed, paraffin-embedded tissue using a SALL4 antibody (Cellmark 6E3 Mouse Monoclonal Antibody, Cell Marque, USA). The BenchMark XT visualization system with enzymatic digestion ISH protease 2 (Ventana, USA) and the iView Blue Detection Kit (Ventana, USA) were employed for visualization. Evaluation of H&E and histochemical specimens was conducted using an Olympus BX46 light microscope. Resection samples were assessed for seminiferous tubule structure, Johnson score, tunica albuginea thickness, interstitial edema, and Leydig cells. Tunica albuginea thickness and seminiferous tubule diameter were measured using the Aperio ImageScope program. Seminiferous tubule damage, interstitial edema, and Leydig cells were scored semiquantitatively on a scale ranging from 0 to 3.

2.5. Statistical analysis

The statistical analysis was conducted using IBM SPSS Statistics Version 20.0. The normal distribution of continuous variables was assessed using the Kolmogorov–Smirnov test.

Continuous variables following a normal distribution were expressed as mean ± standard deviation, and the comparison between the two groups was performed using Student's *t*-test. For continuous variables that did not fit a normal distribution, the data were reported as median (minimum–maximum), and the comparison between groups was carried out using the Mann–Whitney U test. A significance level of 0.05 was adopted for all statistical analyses.

3. Results

3.1. Testis weight and volume

The median testis weight in the study group (163.0 g [133.0 – 183.0]) was significantly lower than the control group (179.0 g [134.0 – 195.0]) ($P = 0.012$). In addition, the median testis volume in the study group (0.95 cm^3 [0.800 – 1.400]) was significantly reduced than the control group (1.100 cm^3 [1.050 – 1.500]) ($P = 0.031$). Furthermore, the median score for seminiferous tubule damage was significantly higher in the study group (1.5 vs. 0.0, $P < 0.001$). The mean tunica albuginea thickness was significantly reduced in the study group (22.3 ± 4.61 μM vs. 87.4 ± 2.67 μM) ($P < 0.001$) (Table 1).

3.2. Changes in the gene expressions of IL-1, IL-4, IL-10, TNF-α, and IFN-γ

The fold changes in the expression levels of the relevant gene in both the study and control groups were calculated using the 2-DDCt analysis and are presented in Figure 2. The delta Ct values for expression analyses of the study and control groups after real-time PCR are shown in Table 2. Notably, the expression levels of IL-4 and IFN-γ genes demonstrated statistically significant increases in fold changes within the study group (n = 10, $P < 0.01$). However, upon analyzing the interchangeability between the groups for other cytokines, no significant difference was detected ($P > 0.1$).

3.3. Vitality analysis using flow cytometry (Annexin V)

A vitality assessment was conducted using flow cytometry. The vitality rates of both groups were similar (43.6 ± 14.2% vs. 45.7 ± 15.6%, $P = 0.709$) (Figure 3).

3.4. Histopathological findings

The tunica albuginea was significantly thinner in the study group (22.3 ± 4.61 μM) than the control group (87.4 ± 2.67 μM) ($P < 0.001$). Seminiferous tubule damage, interstitial edema, and Leydig cells were evaluated semiquantitatively. Seminiferous tubule damage was significantly more pronounced in the study group (Figure 4, $P < 0.001$). While mild interstitial edema was present in the control group (Figure 5A), it was significantly more severe in the study group (Figure 5B, $P = 0.042$). Leydig cell counts were relatively higher in the control group (Figure 5C) than in the study group (Figure 5D, $P = 0.669$). In addition, tunica albuginea thickness was significantly greater in the control group (Figure 5E) than in the study group (Figure 5F) ($P < 0.001$). SALL4 staining was utilized for the detection of germ cell tumors. However, negative SALL4 IHC staining was observed in the seminiferous tubules (Figure 6).

4. Discussion

The present study aims to investigate the effects of RF-EMR on testicular health. This investigation entails the measurement of gene expressions related to inflammatory (IL-1, TNF-α, IFN-γ) and anti-inflammatory (IL-4, IL-10) cytokines in testis tissue, alongside histopathological examination. In addition, the study investigated whether

Table 1. Testis sizes and histopathologic parameters

Parameter	Study group (*n*=10)	Control group (*n*=10)	Statistical test	*p*-value
Testis weight (median [min–max] g)	163.0 (133.0 – 183.0)	179.0 (134.0 – 195.0)	Z=–2.479	0.012
Testis volume (median [min–max] cm^3)	0.95 (0.800 – 1.400)	1.100 (1.050 – 1.500)	Z=–2.174	0.031
Interstitial edema (median [min–max])	1.0 (0.0 – 3.0)	1.0 (0.0 – 1.0)	Z=–2.347	0.042
Leydig cells (median [min–max])	1.0 (1.0 – 1.0)	1.0 (1.0 – 2.0)	Z=–1.549	0.669
Johnson score (median [min–max])	10.0 (10.0 – 10.0)	10.0 (10.0 – 10.0)		N/A
Seminiferous tubule damage (median [min–max])	1.5 (0.0 – 3.0)	0.0 (0.0 – 1.0)	Z=–4.102	<0.001
Tunica albuginea thickness (mean ± SD μM)	22.3 ± 4.61	87.4 ± 2.67	F=3.145	<0.001
Seminiferous tubule diameter (mean ± SD μM)	762.5 ± 83.4	775.7 ± 37.5	F=1.919	0.637
Annexin V (mean ± SD %vitality)	43.6 ± 14.2	45.7 ± 15.6	F=0.006	0.709

Abbreviation: SD: Standard deviation.

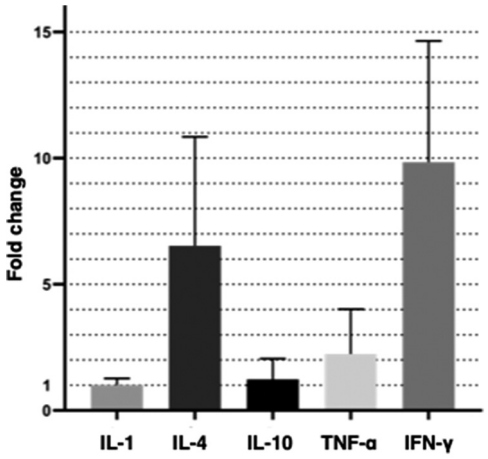

Figure 2. The fold changes in the expression levels of inflammatory cytokines. It shows that interleukin-4 and interferon-γ gene expression fold change increased significantly in the study group (n = 10).

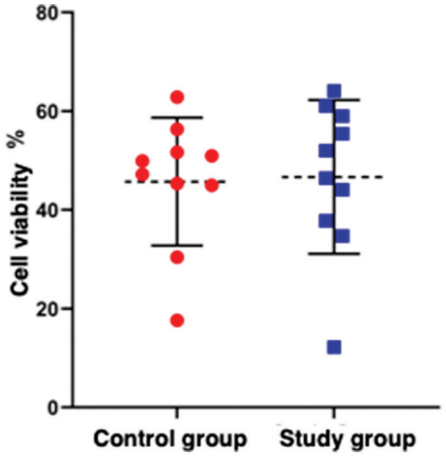

Figure 3. Vitality analysis using flow cytometry (Annexin V). It shows that the viable cell ratios were similar in the study (n = 10) and control (n = 10) groups (43.6% vs. 45.7%, P = 0.709). Student's t-test was used for statistical analysis.

changes in the immune microenvironment induced by RF-EMR exposure might foster a pro-tumorigenic environment conducive to the development of TGCTs. The findings reveal that RF-EMR exposure leads to significant increases in the gene expressions of IL-4 and IFN-γ in the testes. Furthermore, RF-EMR exposure is associated with reductions in testicular volume, weight, and tunica albuginea thickness, alongside significant increases in seminiferous tubule damage and interstitial edema.

It has been demonstrated that RF-EMR significantly weakens antioxidant enzyme activity and increases ROS formation in living organisms.[5] Increased ROS levels precipitate oxidative damage to proteins, lipids, and DNA at the cellular level.[6] Consequently, resultant free

Table 2. Delta Ct values for gene expression analysis of the study and control groups

Group	IL-1	IL-4	IL-10	TNF-α	IFN-γ
Study group					
1	−2.80	−1.00	3.90	3.90	3.89
2	−2.70	−1.80	−0.80	−0.80	−0.78
3	−2.50	0.90	1.00	1.00	0.98
4	−2.70	0.10	0.00	0.00	0.01
5	−2.10	−1.50	−0.20	−0.20	−0.24
6	−2.30	1.50	1.60	1.60	1.57
7	−2.30	−1.30	−2.70	−2.70	−2.67
8	−2.50	0.00	0.50	0.50	0.47
9	−1.80	−0.30	0.70	0.70	0.71
10	−1.80	−0.50	−0.60	−0.60	−0.65
Control group					
1	−2.40	−3.80	5.30	4.40	2.90
2	−2.70	−3.80	−0.10	−1.60	−3.30
3	−2.80	−2.60	0.00	−1.60	−3.10
4	−1.80	−1.60	1.00	1.10	−3.00
5	−2.50	−4.30	−0.90	−0.60	−3.40
6	−1.90	−3.30	−0.30	−2.20	−2.70
7	−2.50	−2.00	1.10	0.20	−3.90
8	−2.60	−2.60	−1.10	−1.00	−2.70
9	−2.30	−2.70	0.30	0.10	−3.00
10	−1.60	−1.30	0.70	−0.20	−2.40

Abbreviations: Ct: Cycle Threshold; PCR: Polymerase Chain Reaction; IL-1: Interleukin-1; TNF-α: Tumor necrosis factor-alpha; IFN-γ: Interferon-gamma.

radicals induce significant changes in the histological and morphological structures of testis and spermatogenic cells.[7] Nisbet *et al.* reported occurrences of testicular vacuolar degeneration, severe necrosis, and exfoliation of the seminiferous epithelia in rats exposed to different levels of RF-EMR ranging from 900 to 1,800 MHz.[8] Similarly, Hasan *et al.* observed histopathologically irregularly shaped testes with inhomogeneous sizes and fewer spermatogenic cell layers, resulting in enlarged lumens in the seminiferous tubules in mice exposed to RF-EMR.[7] Consistent with these findings, our study identifies significant increases in seminiferous tubule damage and interstitial edema in radiation-exposed rats, with a plausible explanation being the degenerative action of RF-EMR on the germinal epithelium and seminiferous tubule.

In a study using 32 male Wistar albino rats, Cetkin *et al.* observed significantly lower testis weight and volume in the RF-EMR exposed group.[9] Similarly, Yahyazadeh *et al.* noted considerably reduced testicular wet weight following exposure to 900 MHz RF-EMR (60 min/day for

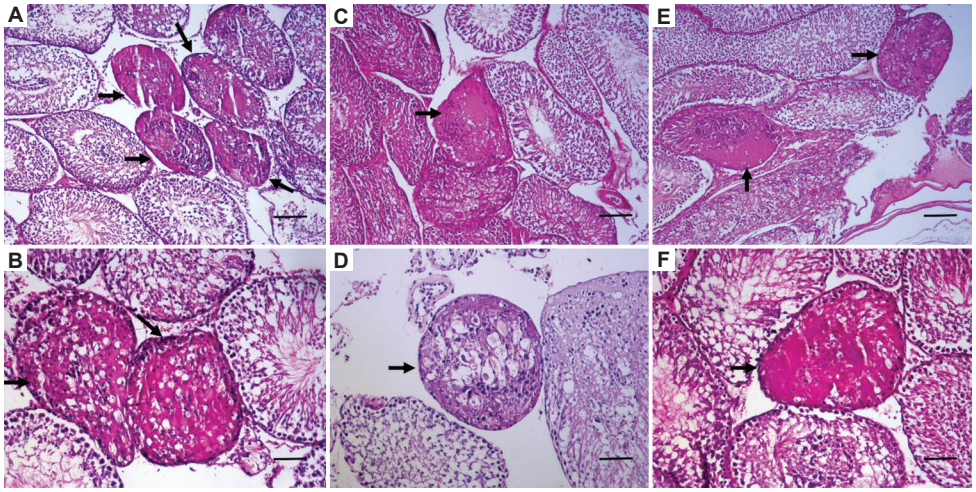

Figure 4. Disorganization, vacuolization, and edema in the seminiferous tubules. Panel A, C, and E indicate H&E magnification ×100, while panel B, D, and F indicate H&E magnification ×200. Scale bars: 100 μm. The arrows indicate disorganization, vacuolization, and edema in the seminiferous tubules. It shows the seminiferous tubule damage induced by radiofrequency electromagnetic radiation in the study group (n = 10).

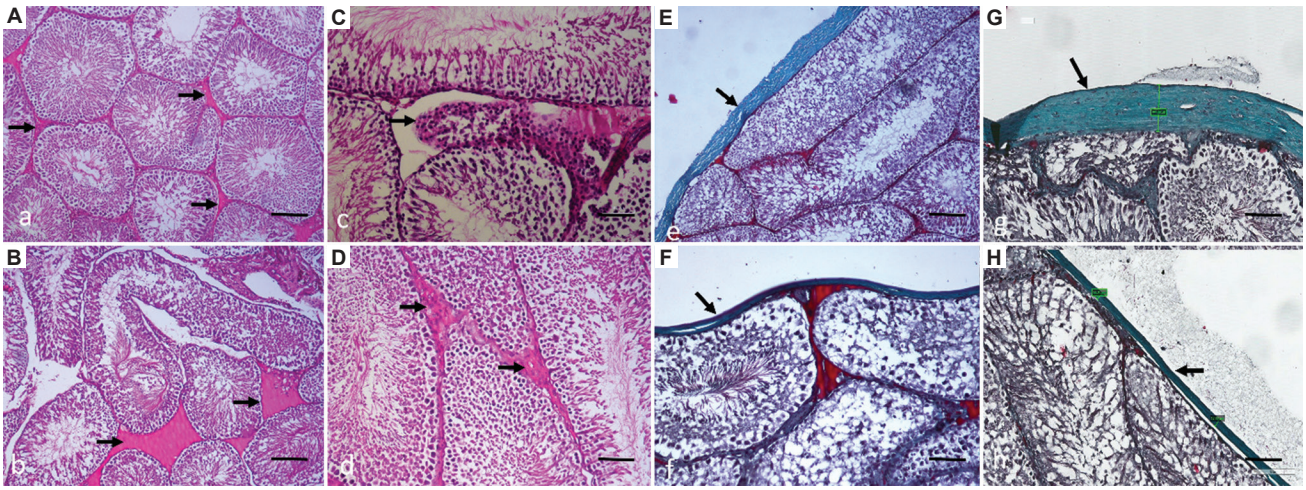

Figure 5. Mild interstitial edema in the control group (n = 10) (A); more severe in the study group (n = 10) (B) (P = 0.042). Leydig cells were relatively higher in the control group (C) than in the study group (D) (P = 0.669). Tunica albuginea thickness was significantly thinner in the study group (F) compared to the control group (E) (P < 0.001). Magnification ×100. Scale bar: 100 μm. The arrows indicate interstitial edema in panels A and B, Leydig cell intensity in panel C and D, and tunica albuginea thickness in panel E, F, G and H. Mann–Whitney U test was used for statistical analysis.

28 days).[10] Consistently, our study observed reductions in testis weight and volume in the RF-EMR exposed group. It is well-established that testicular function and weight are closely intertwined. Agarwal *et al.* reported that cell phones impair testicular function by promoting the formation of free radicals.[11] Therefore, impairments in testicular functions, such as reductions in spermatids and testosterone levels resulting from RF-EMR exposure, alongside changes in endogenous antioxidant enzymes, may contribute to decreases in testicular weights.[10] Moreover, Dasdag *et al.* reported significant reductions in tunica albuginea thickness following exposure to 2.4 GHz radiofrequency radiation,[12] corroborated by our findings of significantly reduced tunica albuginea thicknesses in the RF-EMR exposed group. These histomorphometric findings, including changes in the thickness of tunica albuginea and seminiferous tubule structures, may stem from damage to regulatory mechanisms such as the Na^+/K^+ pump, Na^+/Ca^+ and Na^+/H^+ exchangers, cytoskeleton contractility, and membrane fluidity, which govern cell volume regulation.[12]

The pathogenesis of TGCT remains unclear. This tumor's origin appears to be associated with testicular dysgenesis syndrome, probably initiated during the initial stages of embryogenesis.[13] The most prominent risk factor for TGCT is cryptorchidism, with additional risk factors including a history of TGCT, age, race, and infertility.[13] However, the

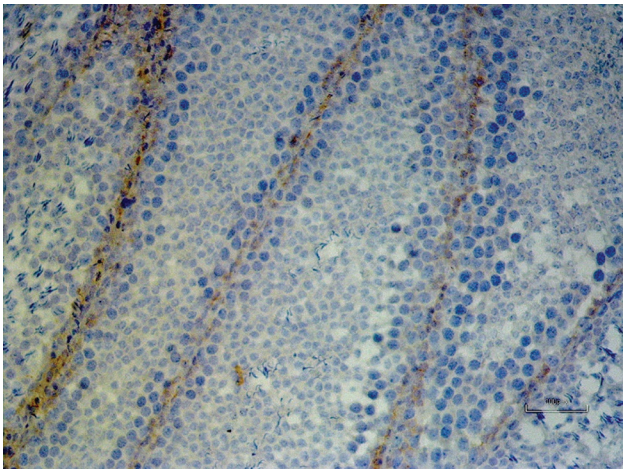

Figure 6. Negative SALL4 immunohistochemical (IHC) staining of seminiferous tubules (IHC × 200). Scale bar: 100 μm.

widely accepted view regarding the risk factors suggests that TGCT development arises from a combination of genetic, environmental, and hormonal factors.[13] There have been suggestions that mobile phones may be associated with the development of TGCT as one of the environmental factors due to their intense and close proximity to the testes.[14] However, the mechanism by which mobile phones could lead to TGCT has not been conclusively demonstrated. Nevertheless, it is acknowledged that inflammatory changes in the testes can be induced by mobile phone-related RF-EMR.[10] Regarding the immune mechanism in TGCT, previous studies have elucidated specific immune cell and cytokine characteristics.[3,4] Various pro-inflammatory cytokines have been implicated in germ cell proliferation, spermatogenetic cell differentiation, TGCT pathogenesis, metastasis, invasion, and neo-angiogenesis.[15] Building upon these data, our study suggests that the inflammation induced by RF-EMR in the testes and the associated immune response may increase the risk of TGCT.

In addition to animal experiments and clinical studies, cell culture investigations have also identified a relationship between TGCT and RF-EMR.[16] Yutong *et al*. examined N9 microglial cells exposed to 2.45 GHz EMF and observed increased levels of activated signal transducers and activators of transcription 3 (STAT3), which subsequently increased the transcription levels of inflammation-related genes, especially inducible nitric oxide synthase (iNOS) and TNF-α. They suggested that exposure to 2.45 GHz EMF could initiate inflammation and malignancy in microglia cells through signal transducer and activator of transcription 3 (STAT3) pathway.[16] STAT proteins comprise various transcription factors that mediate signal transduction from the extracellular environment to the cell nucleus. Notably, the activation of STAT3 is induced by specific cytokines, including IL-4, IFN-γ, growth factors, and oncogenes.[17] Other studies have suggested that IL-4-mediated signaling through STAT contributes to tumor development within the tumor microenvironment.[18-21] Indeed, studies have specifically indicated that dysregulated IL-4 formation is associated with various cancer types.[21,22]

IL-4 is a versatile cytokine and vital for regulating the immune system.[22] Upon IL-4 binding to its cytokine receptor, the ensuing activation of cell growth mediators, resistance to apoptosis, gene activation, and differentiation occur.[22] The significance of IL-4 as a promoter of tumor-initiating/cancer stem cell (CSC)-like cells has been demonstrated across various cancers. Elevated levels of IL-4 (generally generated by tumor-infiltrating lymphocytes) have been verified in advanced-stage prostate cancer (PC) patients.[23] *In vitro* studies with PC cell lines have demonstrated that IL-4 stimulates NF-κB and androgen receptors in a ligand-independent manner.[24] In colon cancer, CD133-positive tumor-initiating cells exhibit autocrine IL-4 signaling, leading to the upregulation of the anti-apoptotic protein Survivin, a target of the STAT-6 pathway.[25] In the pancreatic cancer cell line Capan-1, the knockdown of IL4Rα results in reduced cell growth, decreased anchorage-independent colony size, and inhibition of migration.[26] Similarly, in breast cancer cells, an antagonist of the IL-4 receptor IL4Rα was able to reduce the number of CD44+/CD24- CSC-like cells.[27] However, there is currently no study in the existing literature that conclusively proves the relationship between IL-4 and the development of TGCT. Our research revealed that exposure to RF-EMR increased IL-4 gene expression levels in rats. Although we lack robust evidence, this finding prompts the question: Could IL-4 exhibit a similar relationship with TGCT as observed with the aforementioned tumors?

The IFN-γ cytokine is predominantly generated by activated T lymphocytes and natural killer cells.[28] Although IFN-γ is effective against microbial infections, it also plays essential roles in numerous diseases, especially various types of cancers. In the past, IFN-γ was recognized solely for its antitumor properties.[28] The cytotoxic effects of IFN-γ, especially against tumor cells, have been extensively demonstrated in numerous studies. Subsequently, the pro-tumor effects of IFN- γ began to emerge. The discovery that IFN-γ promotes the expression of inhibitory molecules such as programmed cell death ligand 1 (PDL1), PDL2, indoleamine 2,3-dioxygenase 1 (IDO1), iNOS, FAS, and FAS ligand (FASL), all of which limit antitumor immunity, has raised concerns regarding the use of IFN-γ-modulating cancer immunotherapies.[29] Benci *et al*. reported that initial exposure to IFN-γ primes other factors to promote antigen presentation, T cell priming and activation, and tumor cell killing. Despite that, prolonged

exposure to IFN-γ transforms these supportive factors into adversaries, facilitating pro-tumorigenic effects via immunosuppression, angiogenesis, and tumor cell proliferation.[30] Similar to other tumors, investigations into the relationship between IFN-γ and tumor development, as well as its potential as a therapeutic target, were conducted for TGCT. Schweyer *et al.* analyzed TGCT cell lines (NTERA and NCCIT) and concluded that although they expressed and secreted IFN-γ, they were resistant to endogenous IFN-γ, as neutralization of IFN-γ with a specific antibody did not affect the proliferation and/or degree of apoptosis of the tumor cells.[28] Klein *et al.* examined the immune cellular microenvironment in testis biopsy specimens from patients with normal spermatogenesis, hypo-spermatogenesis with lymphocytic infiltration, GCNIS, and seminoma.[4] Alongside pro-inflammatory (IL-1b, IL-6, and TNF-a), anti-inflammatory (TGF-b1), Th-1-driven (IL-2) cytokines, and chemokines (CXCL-13, CXCL-10, and CCL5), high levels of IFN-γ transcripts were observed within immune cell infiltrates of GCNIS and seminoma samples.[4] Despite extensive research, the relationship between RF-EMR and the development of TGCT remains unestablished in the literature. The significant increase in the IFN-γ gene expression levels that we detected following RF-EMR exposure may potentially influence the development of TGCT.

The limitation of our work was the examination of a limited number of inflammatory markers due to budget constraints. Current budget restrictions prevented us from additional IHC analyses to observe and compare cytokine expression in tissue and protein levels using the ELISA method. However, our study boasts several strengths. Firstly, our experimental setup comprehensively examines both the IHC changes induced by RF-EMR in testicular tissue and the gene expressions of inflammatory cytokines. In addition, the identification of increased expression of IFN- γ and IL-4 genes, which have proven to influence the progression of various cancer types, provides valuable insights for further studies exploring the potential relationship between RF-EMR exposure and the development of TGCT.

5. Conclusion

RF-EMR induces structural, histopathological, and inflammatory toxic effects on testes. RF-EMR significantly reduces testes' weight and volume, increases seminiferous tubule damage and interstitial edema, and decreases tunica albuginea thickness. However, it also significantly increases the gene expression levels of IL-4 and IFN-γ, which are inflammatory cytokines that are potentially associated with TGCT development in testicular tissue. Therefore, these cytokines expressed following RF-EMR exposure may serve as regulators of TGCT initiation, offering a viable potential therapeutic target in combination with current treatment options. Nevertheless, future well-designed studies are imperative to substantiate our findings.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

The study received ethics approval from the Institutional Animal Ethics Committee (Protocol Authorization Number: 5-2-2019).

Consent for publication

Not applicable.

Availability of data

Data used in this work is available from the corresponding author upon reasonable request.

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Appendix

Table 1. Primers sequences used for PCR.

Gene name	Primer sequence
Il-1 (NM_008361)	
Il-1-F	TGGACCTTCCAGGATGAGGACA
Il-1-R	GTTCATCTCGGAGCCTGTAGTG
Il-4 (NM_021283)	
IL-4-F	ATCATCGGCATTTTGAACGAGGTC
IL-4-R	ACCTTGGAAGCCCTACAGACGA
Il-10 (NM_010548)	
Il-10-F	CGGGAAGACAATAACTGCACCC
Il-10-R	CGGTTAGCAGTATGTTGTCCAGC
TNF-α (NM_013693)	
TNF-α-F	GGTGCCTATGTCTCAGCCTCTT
TNF-α-R	GCCATAGAACTGATGAGAGGGAG
IFN-γ (NM_008337)	
IFN-γ-F	CAGCAACAGCAAGGCGAAAAAGG
IFN-γ-R	TTTCCGCTTCCTGAGGCTGGAT

Abbreviations: IL-1: Interleukin-1; TNF-α: Tumor necrosis factor-alpha; IFN-γ: Interferon-gamma; PCR: Polymerase chain reaction.