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Original Research

Stress-Responsive MAPK Signaling Pathway with Proliferation and Apoptosis in the Rat Testis After 2100 MHz Radiofrequency Radiation Exposure

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ABSTRACT

Objective: Mobile phone technology has progressed quickly in recent years. Cell phones operate using radiofrequency radiation (RFR), and the complete biological impacts of RFR remain unidentified. Thus, we aimed to investigate the potential effects of 2100 MHz radiofrequency radiation exposure on the stress-responsive JNK/p38 MAPK pathway, apoptosis and proliferation in rat testis.

Methods: RFR groups were created with 2100 MHz RFR exposure for acute (2 h/day for 1 week) and chronic (2 h/day for 10 weeks) periods. Sham groups were kept under identical conditions without RFR. The cell apoptosis and histopathological changes in testis were evaluated. Immunolocalization of PCNA, active caspase-3, Bcl-xL, p-JNK and p-p38 were analyzed by immunohistochemistry, the total protein expressions were identified by Western blot.

Results: There were no differences between RFR and sham groups by means of histopathology and TUNEL analysis. Also, the expression levels and the immunolocalization patterns of PCNA, active caspase-3 and Bcl-xL proteins were not altered. p-JNK and p-p38 protein expressions were prominently elevated in acute and chronic RFR groups.

Conclusion: In conclusion, 2100 MHz RFR exposure had no considerably deleterious consequences on cellular proliferation and apoptosis processes in rat testis. However, increased expression of stress-activated protein kinases, p-JNK and p-p38, suggests the involvement of the MAPK signaling pathway as a critical (may be detrimental) cellular response.

Keywords: Apoptosis, MAPK pathway, proliferation, radiofrequency radiation, rat, testis

INTRODUCTION

Throughout the years, the technologies of our daily devices have undergone upgrades, leading to significant advancements in the world due to the rapid progress of the technology. Mobile phones are one of the most commonly used technological devices in today's world. However, they work with radiofrequency radiation (RFR). Thus, it is crucial to investigate any possible negative impacts that mobile phones may have on human health. The quantity of studies in this area has drastically expanded during the last century. Despite the fact that the mechanisms

underlying RFR's biological effects have not yet been fully elucidated, RFR has been categorized as a potential carcinogen by the International Agency for Research on Cancer in 2017 [1].

The majority of studies indicated that RFR exposure and unfavorable biological consequences are directly correlated [2-4]. The studies report that RFR increases reactive oxygen species (ROS) [3], causes fatigue and headache [4], affects brain signals [5], decreases sperm count [6] and causes testicular apoptosis [7]. Oxidative stress (OS) particularly takes a special attention related to RFR exposure recently as it has a significant potential to have these biological side effects. Oxidative stress arises because of increased levels of ROS or oxidants. The increased free radical production or weakening of the antioxidant defense system can negatively change lipids, proteins, DNA and trigger a number of human diseases. Thereafter, ROS acts as a double-edged sword. They also function as key signal molecules in fertility related biological processes including fertilization, capacitation and hyperactivation of sperm and fusion of oocyte and sperm plasma membrane [8]. For instance, excess ROS can induce the dysfunction of sperm through motility loss, DNA and lipid peroxidation [9]. In an ultrastructural study in which rats were applied to long-term (3 months) low-dose RFR, no significant alteration was found in the testicular weight and seminiferous tubule diameters; however, vacuolization in Sertoli cells was reported [10]. Moreover, there have been studies indicating that carrying a mobile phone adjacent to testis may have detrimental effects on sperm concentration and motility [7]. Besides, Leydig cells' secretory activity (serum testosterone level) was found to be reduced after 220 MHz RFR exposure in male adult rats [2]. It was also noted that male rats exposed to Wi-Fi RFR for a year experienced decreased epididymal and seminal vesicles weight, a decrease in tunica albuginea thickness and seminiferous tubule diameter, as well as an increase in sperm head abnormalities [11].

Main Points:

- 2100 MHz RFR exposure does not alter testicular cell proliferation and apoptosis
- Expression of stress-activated MAPKs were increased after exposure to 2100 MHz RFR
- A special attention is needed to the molecular mechanism of MAPK signaling pathway

It is known that OS induces apoptosis in cells by activating multiple signaling pathways [12]. The mitogen-activated protein kinase (MAPK) cascade is defined as a critical pathway for transmitting stress-related stimuli [13]. Growth factors, hormones, radiation, carcinogenic agents and inflammation are some examples of stimuli that might activate the MAPK cascade. This activation results in different biological reactions like cell division, differentiation, migration and apoptosis [13]. The MAPK family is consisted of proteins p38, c-Jun NH2-terminal kinases (JNKs), and extracellular signal-regulated kinases (ERKs) [13]. The growth stimuli can activate ERK1 and ERK2 and contribute to cell growth; however, JNK and p38 MAPKs, which are stimulated by some environmental stress and inflammatory factors, stimulate cell apoptosis and the inhibition of growth [14]. The mammalian testis has a variety of MAPK isoforms. MAPKs function in spermatogenesis, maturation and capacitation of sperm [15] and also in the fertilization of oocytes [16].

Our research group have recently reported that p38/JNK-mediated MAPK signaling was triggered significantly in the rat testis after 900 MHz RFR exposure [17]. However, it is unknown whether the p38/JNK-mediated MAPK signaling is also involved in the rat testis after 2100 MHz RFR exposure, which is used in global mobile communication systems (GSM). Currently, it is clear that the JNK/p38 MAPK pathway is important in biological reactions in response to ROS. Since the effects of the JNK/p38 MAPK cascade on cell survival or cell death events have not been completely elucidated, here our purpose is to examine the proliferation/apoptosis balance and the JNK/p38 MAPK proteins in the rat testis after acute and chronic 2100 MHz (Universal Mobile Telecommunication System, UMTS, frequency, UMTS-2100) RFR exposure.

MATERIALS AND METHODS

Study Groups

The experimental animal protocols utilized in this study received approval from the Akdeniz University Local Ethics Committee for Animal Experiments (protocol number: 758/2018.10.07). Male Wistar albino rats weighing 250-300 g were categorized as Group 1 (sham control group for one week-acute sham: aSh), Group 2 (sham control group for 10 weeks-chronic sham: chSh), Group 3 (2100 MHz RFR exposure group for one week-acute RFR: aRFR) and Group 4 (2100 MHz RFR exposure group for 10 weeks-chronic RFR: chRFR) (n = 6/each group). Rats were kept in plexiglass tubes at a distance of 10 cm radially around

the 2100 MHz radiofrequency (RF) emitting antenna. Rats in Groups 1 and 3 were held in plexiglass tubes for one week (2 h/day, 5 days per week). On the other hand, rats in Groups 2 and 4 were held in plexiglass tubes for 10 weeks (2 h/day, 5 days per week). Radiofrequency simulator device was retained in OFF state for the control groups and ON state for RFR exposure groups throughout the experiments. Animals (4 rats/cage) were kept in a 12 h light/dark cycle during the experiment periods and provided tap water and commercial rat chow.

Radiofrequency Radiation Treatment

Except for the 2100 MHz RFR frequency and the distance of the rats from the antenna, the details of the experimental setup and RFR application were as previously reported [17]. An RF generator which produce 2100 MHz RFR (2100 MHz UMTS Simulator; Everest Corp., Adapazari, Türkiye) was used to represent the exposure of the UMTS. 2100 MHz used as carrier frequency and the pulse width was 0.577 ms. The modulation frequency was 217 Hz, and the generator's power range was 0-2 W. The electric-field potency over the rat's head at a distance of 10 cm from the antenna was 35.2 V/m for 2100 MHz when the experiment was in "signal on" status.

Using a finite integration technique (FIT) program, CST Microwave Studio (3DEXPERIENCE, Dassault Systemes, Hamburg, Germany), dosimetry simulations were performed. The FIT was established by Weiland [18]. The electrical features of the testis tissue of the simulation model rats were measured at the operating frequency. At 2100 MHz, the average specific absorption rate (SAR) for the whole-body was 0.16 W/kg. On average, the SAR value for the testis was 0.0347 W/kg.

Sample Collection and Histopathological Analysis of the Testis

Rat testicular tissues were dissected at the end of the experiments as described previously [17]. The potential consequences of acute and chronic 2100 MHz RFR exposure on testis histopathology were investigated after sections were deparaffinized and stained with periodic acid-Schiff (PAS).

Immunohistochemistry

The immunolocalization patterns of proliferating cell nuclear antigen (PCNA), active caspase-3, Bcl-xL, phosphorylated-JNK (p-JNK) and phosphorylated-p38 (p-p38) proteins were carried out in the paraffin sections of testis tissues. PCNA (cat#2586S; Cell Signaling, 1:600), Bcl-xL (cat#2764S; Cell Signaling, 1:300), active caspase-3 (cat#9661S; Cell Signaling, 1:300), p-JNK (cat#44682G; Thermo Fisher Scientific, 1:200) and p-p38 (cat#4511S; Cell Signaling, 1:100) antibodies were used as primer antibodies. Immunohistochemistry protocol is described in more detail in our recent paper [17]. The threshold quantification of the immunohistochemical images was further evaluated blindly for the localization patterns by using ImageJ version 1.46 (NIH)

SDS-PAGE and Western Blotting

The membranes were incubated with primary antibodies in TBS-T and 5% nonfat dry milk overnight (cat#2586S, PCNA, Cell Signaling, 1:1000; cat#2764S, Bcl-xL, Cell Signaling, 1:1000; cat#9661S, active caspase-3, Cell Signaling, 1:500; cat#44682G, p-JNK, Thermo Fisher Sci., 1:1000 and cat#4511S, p-p38, Cell Signaling, 1:500). The details for the Western blot protocol were previously presented [17]. The optical densities of the Western blot bands were standardized to OD values of beta actin, and the relative amounts of proteins were analyzed by ImageJ version 1.46 (NIH).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

The apoptotic cells were identified by TUNEL method with a commercial TUNEL kit (cat#1684809, In situ Cell Death Detection Kit, AP, Roche Diagnostics, Germany) as published previously [17]. The number of TUNEL-positive/negative seminiferous tubules were counted blindly and the percentages of positive seminiferous tubules were further calculated for experimental groups (% apoptotic index).

Statistical Analysis

The statistical analysis between acute and chronic RFR periods versus control groups of the related week was compared by SigmaStat 3.5 (Systat Software, USA). All normally distributed data were analyzed by one-way analysis of variance (ANOVA). However, data were compared using Kruskal-Wallis test followed by Dunn's test, or the Mann-Whitney U test if the data are not normally distributed. The results were presented as mean ± SEM. The statistical significancy of the data was accepted as $p < 0.05$.

RESULTS

Histopathological Evaluation of the Testis Tissues After 2100 MHz RFR Exposure

Leydig cells, acrosome structures, and seminiferous tubule basement membranes were clearly observed in the testis

sections from all experimental groups by PAS staining (Figure 1). However, no prominent histopathological differences were detected for acute or chronic RFR groups compared to control groups.

Proliferation and Apoptosis of the Testicular Cells After 2100 MHz RFR Exposure

Immunolabeling cells with PCNA revealed that PCNA-positive cells were similarly localized as a single layer along the basement membrane of the basal compartment of the seminiferous tubules in RFR versus related control groups (Figure 2A-D). Immunoreactivity for PCNA was observed in the spermatogonia nuclei and some spermatocytes. In line with no differences in the distribution patterns, no significant alterations were observed in neither the PCNA staining intensities by ImageJ analysis (Figure 2E) nor the total protein levels by Western blotting between the control and RFR groups (Figure 6A, B).

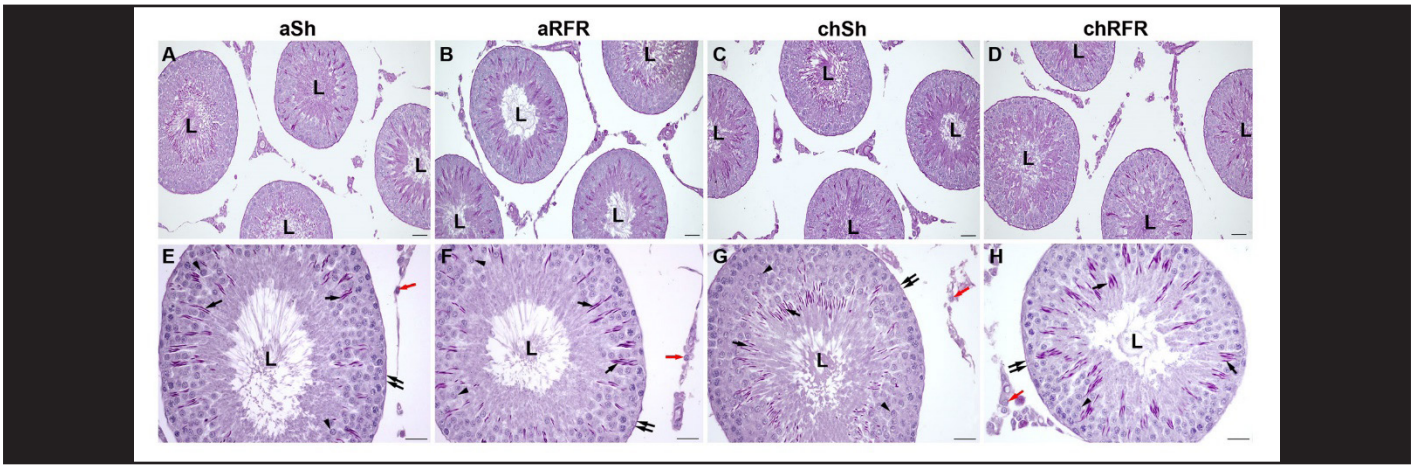

Figure 1. PAS staining in the rat testis tissues of the control and RFR groups. Please observe PAS-positive elongated spermatids (arrows), basement membrane (double arrows), acrosomal caps (arrow heads) and Leydig cells (red arrows). Acute control (aSh): A, E; acute RFR (aRFR): B, F; chronic control (chSh): C, G; chronic RFR (chRFR): D, H; lumen (L). Scale bars present 50 µm

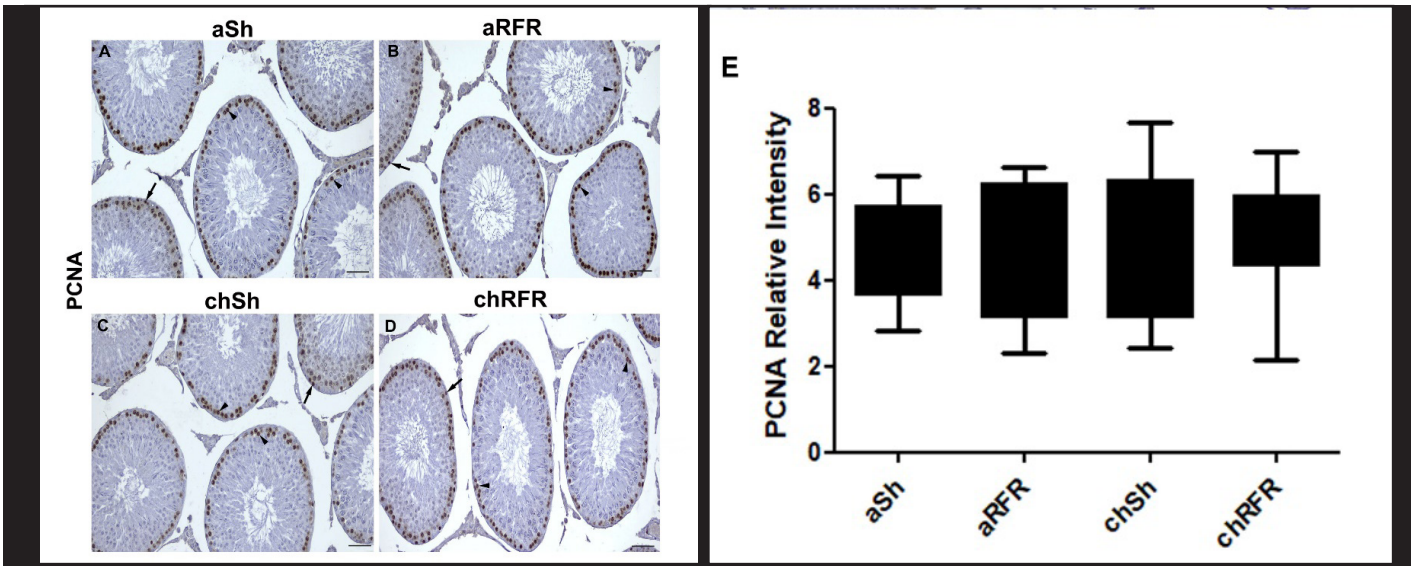

Figure 2. PCNA immunostaining in the rat testis tissues of the control and RFR groups (A-D) and the data for the ImageJ analysis of the immunostaining (E). Spermatocytes (arrow heads), spermatogonial cells (arrows). Acute control (aSh): A; acute RFR (aRFR): B; chronic control (chSh): C; chronic RFR (chRFR): D. Scale bars present 50 µm

The active caspase-3 immunostaining was identified mostly in postmeiotic germ cells, predominantly in round spermatids in the rat testis (Figure 3A-D). However, immunostaining of Bcl-xL was detected in the cytoplasmic areas of round spermatids and spermatocytes (Figure 3E-H). Additionally, Bcl-xL was expressed in elongated spermatids and mature spermatozoa (Figure 3E-H). As observed with PCNA immunostaining, the intensity and distribution patterns of active caspase-3 (Figure 3I), an enzyme responsible for the majority of proteolysis during apoptosis, and Bcl-xL (Figure 3J), as an anti-apoptotic protein, were not noticeably different in RFR groups with ImageJ analysis. The analysis of the protein bands by Western blot also indicated that the total protein expressions of both active caspase-3 and Bcl-xL proteins in the testicular tissue lysates from RFR-exposed groups were not altered compared to those in the testis tissues from control groups (Figure 6A, C, D).

In addition to these markers, apoptosis was evaluated by the TUNEL assay. TUNEL-positive cells were determined particularly in spermatocytes, spermatogonia and Leydig cells. Our data indicated that there was no increase in the apoptotic activity revealed by the TUNEL method in the testis tissues of the RFR groups (Figure 3K). Therefore, the effects of 2100 MHz RFR exposure on spermatogenesis could not be obviously identified as a death and/or an alteration in the testicular cell proliferation.

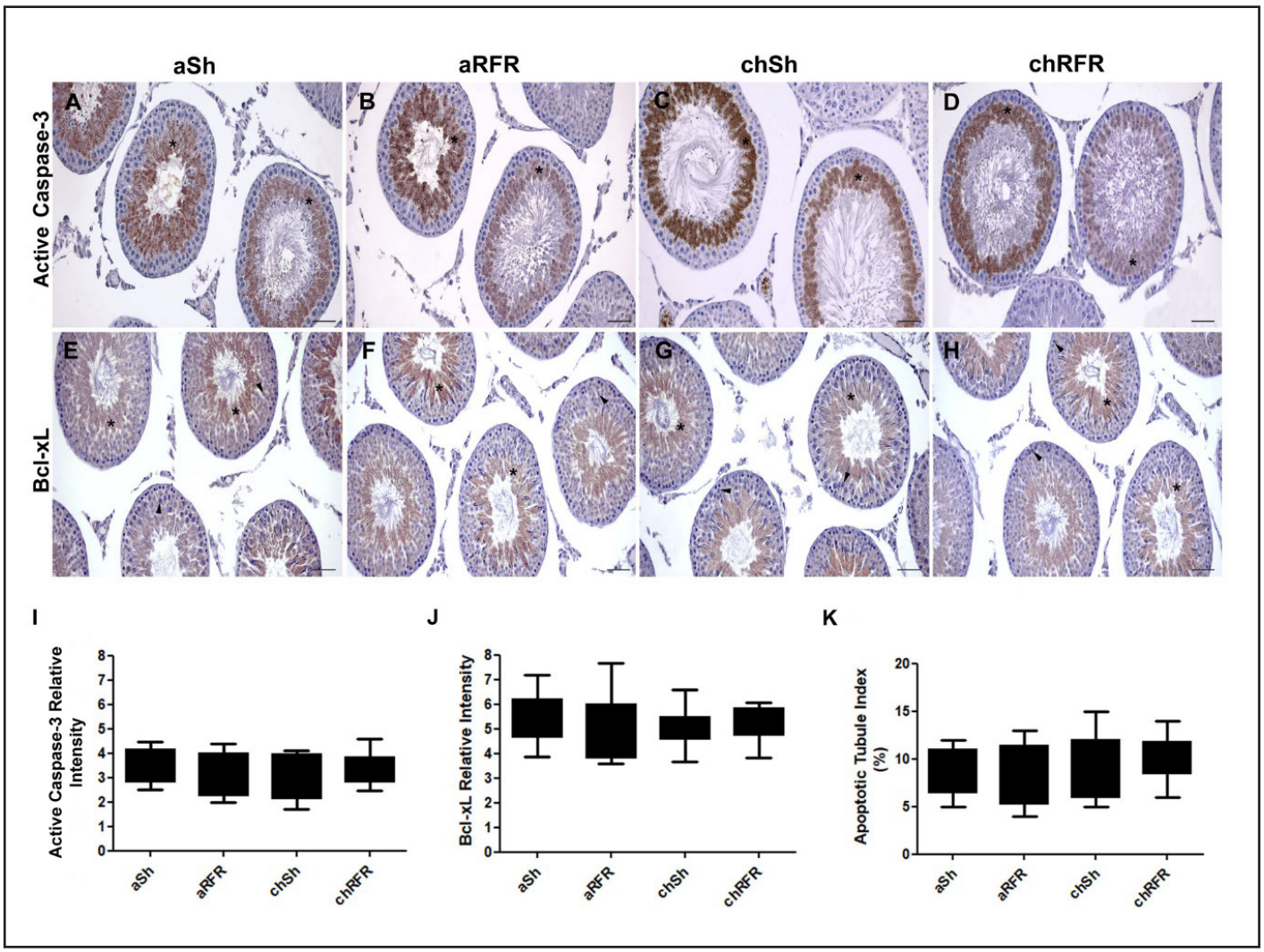

Figure 3. Active caspase-3 (A-D), Bcl-xL (E-H) immunostaining, and the apoptotic tubule index (K) in the rat testis tissues of the control and RFR groups. ImageJ analyses of active caspase-3 (I) and Bcl-xL (J) immunostaining are also presented. Postmeiotic germ cells (asterisks), spermatocytes (arrow heads). Acute control (aSh); acute RFR (aRFR); chronic control (chSh); chronic RFR (chRFR). Scale bars present 50 μm

Altered Expression of JNK and p38 Proteins After 2100 MHz RFR Exposure

We detected a marked testicular expression for p-p38 and p-JNK MAPK proteins in RFR groups. Although p-JNK immunoreaction was detected in the nuclei of spermatocytes, spermatogonia, and the heads of elongated and round spermatids in some seminiferous tubules and also in Leydig cells (Figure 4A-H), a considerable increased expression of p-JNK protein in both acute ($p < 0.001$) and chronic ($p < 0.001$) RFR groups were detected by ImageJ analysis (Figure 4I). Similarly, Western blot analysis indicated an increased total protein level of p-JNK protein in the testis lysates (Figure 6A, E).

p-p38 expression was similarly located in the spermatocyte nuclei, elongated spermatids, round spermatids, and some of the Leydig cells (Figure 5A-H). As of p-JNK, a dramatic increase in the staining intensity for p-p38 in the testis sections from RFR groups was determined by ImageJ analysis ($p < 0.001$) (Figure 5I). Our Western blot data further confirmed that exposure to 2100 MHz RFR resulted in an increased expression of p-p38 protein for both acute and chronic RFR groups (Figure 6A, F).

DISCUSSION

Modern society has become accustomed to using any technological device or product to perform almost anything. In this line, mobile phones have a crucial role in our daily lives as they are the easiest form of communication, and they have become an indispensable part of our lives. Although there is widespread concern about the risks of RFR exposure from mobile phones on human health, perceptions of risk associated with other sources of electromagnetic fields must be investigated. It has been suggested that RFR may cause oxidant damage [3], brain electrical activity changes [5], DNA damage [19] *etc*. RFR may also have detrimental effects on the testis tissues [17, 20]. Therefore, we investigated the consequences of 2100 MHz RFR exposure on the rat testis by means of cell proliferation, apoptosis and the MAPK signaling pathway including p-p38 and p-JNK stress-activated protein kinase (SAPK) proteins.

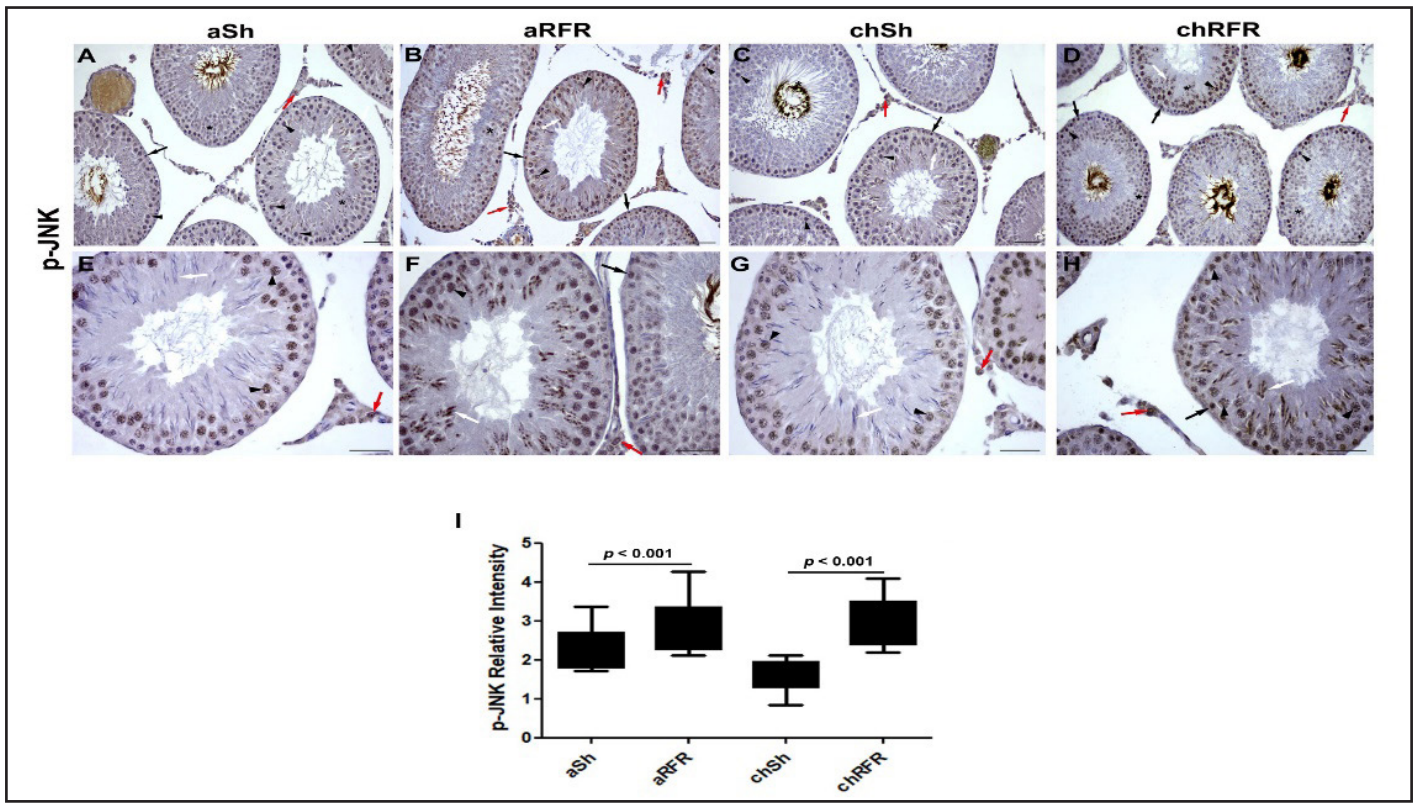

Figure 4. p-JNK immunostaining in the rat testis tissues of the control (A, E, C, G) and RFR (B, F, D, H) groups and their ImageJ analyses (I). p-JNK-positive spermatocytes (arrow heads), spermatogonial cells (arrows), round spermatids (asterisks), Leydig cells (red arrows) and elongated spermatids (white arrows). Acute control (aSh); acute RFR (aRFR); chronic control (chSh); chronic RFR (chRFR); L, lumen. Scale bars present 50 µm

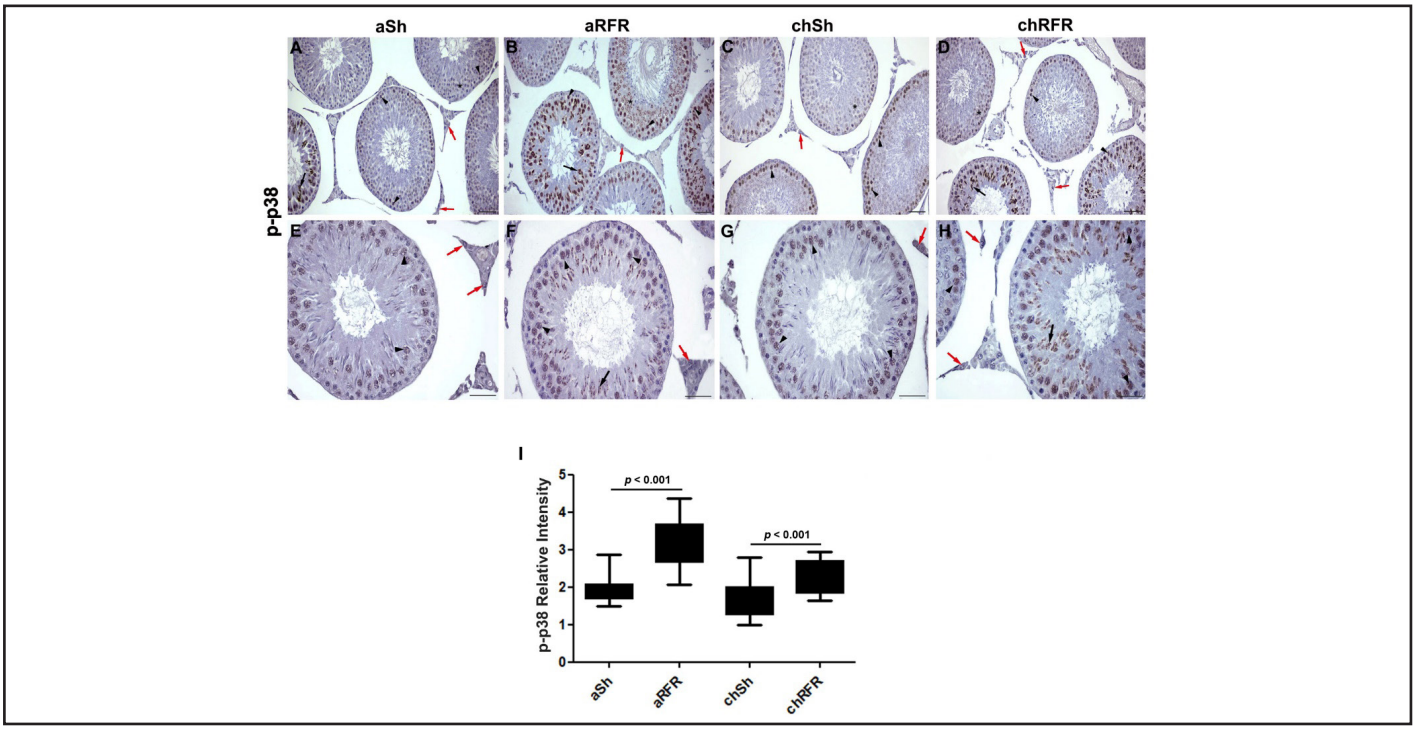

Figure 5. p-p38 immunostaining in rat testis tissues of control (A, E, C, G) and RFR (B, F, D, H) groups and their ImageJ analyses (I). p-p38-positive round spermatids (asterisks), spermatocytes (arrow heads), Leydig cells (red arrows) and elongated spermatids (arrows). Acute control (aSh); acute RFR (aRFR); chronic control (chSh); chronic RFR (chRFR); L, lumen. Scale bars present 50 µm

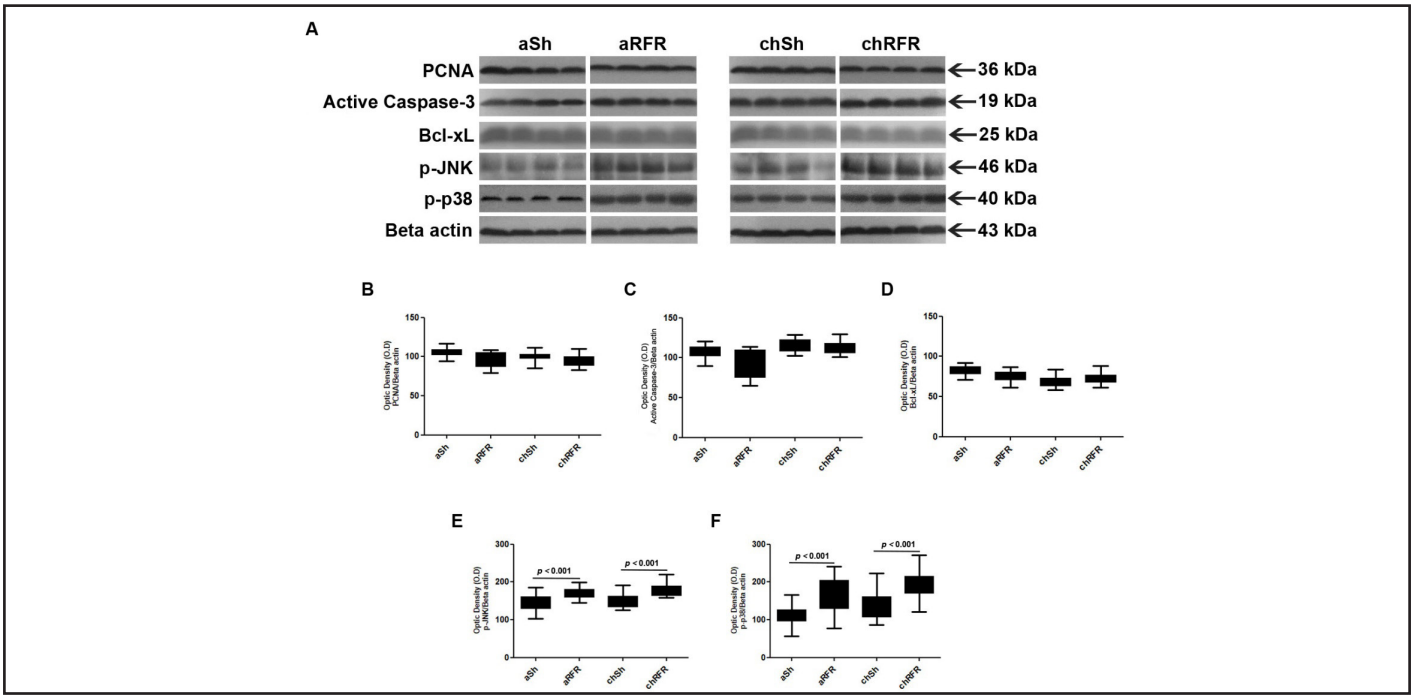

Figure 6. Total protein analysis of the testis lysates of the control and RFR groups by Western blot (A). Please observe the bands for PCNA (36 kDa), active caspase-3 (19 kDa), Bcl-xL (25 kDa), p-JNK (46 kDa) and p-p38 (40 kDa) proteins. Total protein levels of the target proteins are normalized to beta-actin (43 kDa) as a loading control and graphed (B-F). Acute control (aSh); acute RFR (aRFR); chronic control (chSh); chronic RFR (chRFR)

The interaction of RFR and cell proliferation has been a very curious subject. Unfortunately, there are very few studies in the current literature providing the different results with conflicting data [21, 22]. The reports on testis, skin, and brain tissues indicated that PCNA expressions did not change in the RFR groups [17, 21, 23]. In contrast, some studies revealed that PCNA expressions were reduced in testicular and liver tissues of RFR groups [22, 24]. However, we found no significant differences in PCNA expression and its distribution patterns in the rat testis after acute and chronic 2100 MHz RFR exposure in terms of both Western blot and immunohistochemistry methods.

On the other hand, we also aimed to investigate the consequences of RFR administration on testicular apoptotic and antiapoptotic factors in addition to cell proliferation after 2100 MHz RFR exposure. The antiapoptotic (Bcl-xL and Bcl-2) and proapoptotic (Bax and Bad) Bcl-2 protein family have significant functions in the survival and death of cells [25]. Our data clearly indicated there was no alteration in testicular cell apoptosis as observed for cell proliferation in RFR groups. Besides, neither the expression patterns nor the protein expression of active caspase-3 and Bcl-xL proteins were statistically different from the control groups in RFR groups. The TUNEL assay was also performed alongside the active caspase-3 and Bcl-xL proteins. However, it did not reveal any change in terms of apoptotic cell death in RFR groups. In fact, we previously reported that apoptosis in the rat testis was increased after 900 MHz exposure for one week by TUNEL analysis. However, no difference was observed after 10 weeks [17]. It was reported that there wasn't any alteration in the testicular cell apoptosis of 5-weeks-old rats with 2.45 GHz RFR exposure for three weeks (2h/day), but an increase was observed in the testis tissues of 6-weeks-old rats [26]. Interestingly, it has been shown that apoptosis was increased in the offspring rat testis after 900 MHz RFR for 1 h/day through days 13-21 of pregnancy [27]. In another study with the administration of 900 MHz RFR (SAR value: 0.07-0.57 W/kg) for 2 h/day during 10 months, no obvious difference was identified in the quantification of cells stained positively with active caspase-3 in testis tissue between experimental groups [28]. However, Guo and colleagues demonstrated that the number of active caspase-3-positive cells and testicular protein level were significantly higher after exposure to 220 MHz RFR (SAR value: 0.014 W/kg) for a month (1 h/day) [2]. Our data for active caspase-3 supports the data that of Dasdag and colleagues [28]. Besides, there are limited number of studies about the Bcl-2 proteins after RFR exposure on the testis. Some of the studies with rat or mouse testis indicated that Bcl-2 or Bcl-xL expressions were decreased after RFR exposure [17, 29, 30] although some other publications, including our study, revealed that there wasn't any remarkable alteration in Bcl-xL expression [17, 31, 32]. In fact, our previous data has shown a decreased expression level of Bcl-xL protein in the acute period; however, we reported no significant difference after chronic 900 MHz RFR exposure in the rat testis [17]. The active caspase-3 protein was not changed after acute period, but a dramatic reduction was observed in the 900 MHz chronic RFR group by means of the Western blot technique. However, it was not detected in the ImageJ analysis of the immunohistochemical images [17]. Therefore, the usage of the different RFR frequencies, exposure duration periods, SAR values, age or situations such as pregnancy and also the sensitivity of the analysis methods in different species may be potential reasons why the results were altered between several research groups.

JNK and p38 SAPK proteins can affect apoptosis via Bcl-xL [33]. Therefore, the consequences of 2100 MHz RFR on p-p38 and p-JNK proteins were investigated in line with Bcl-xL expression in the rat testis tissue in the current study. Our data clearly revealed that the expressions of both proteins were significantly higher in acute and chronic RFR groups. In a study in which 30 mW/cm^2 microwave radiation was used, p-p38 and p-JNK expressions were increased in the rat testis after a 6-hours exposure [34]. However, there were not any significant differences in the 7-day exposure group. In a similar manner to 2100 MHz RFR, we previously demonstrated that the expression of p-p38 and p-JNK proteins were increased considerably after 900 MHz RFR exposure [17]. It is clearly observed that the activation of a MAPK cascade occurs in an experimental setup after 2100 MHz RFR exposure in rat testis with no changes in cell proliferation or apoptosis.

Limitations

In fact, one can suggest that the various sperm parameters, including sperm motility and concentration, should also be examined in future studies in order to exclude the possibility that 2100 MHz RFR exposure causes no effects on male infertility.

CONCLUSION

Here we report that exposure to 2100 MHz RFR for up to 10 weeks with a low SAR value (up to 2 h daily use) does not result in an obvious harmful effect on rat testis by means of testicular cell proliferation and apoptosis. However, the expressions of

stress-activated protein kinases, p-p38 and p-JNK, were clearly increased in the rat testis tissues. Thus, a special attention is needed to elucidate this molecular mechanism of action and its undesirable side effects.

Funding Support

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Data Availability

The data supporting the findings of this study can be obtained from the corresponding author upon a reasonable request.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Ethics Approval

The experimental animal protocols utilized in this study received approval from the Akdeniz University Local Ethics Committee for Animal Experiments (protocol number: 758/2018.10.07).

Author Contributions

Conception-H.E., L.S.; Design-H.E., L.S.; Supervision-L.S.; Fundings-L.S.; Materials-H.E.; Data Collection or Processing-H.E., B.S., G.G.T., S.O.; Analysis or Interpretation- H.E., B.S., G.G.T., S.O., L.S.; Literature Review-H.E., L.S.; Writing-H.E., L.S.; Critical Review-L.S.

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