

Can 900 MHz and 2100 MHz radiofrequency radiation exposure induce endoplasmic reticulum stress and apoptosis in rat thymus?

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ABSTRACT

Objectives: Electrical appliances are source of radiofrequency radiation (RFR). The effects of RFR on the organism are not fully understood. Endoplasmic reticulum (ER) stress is appeared by the accumulation of misfolded proteins in ER lumen. The aim of this study was to investigate the effects of 900 and 2100 MHz RFR exposure on the ER stress pathway in rat thymus.

Methods: Rats were divided into six groups: 1 week (w) and 10 w Sham rats were kept in plexiglass tubes for 2 hours/day without RFR, experiment groups were created as 1-w (acute) and 10 w (chronic) rats which exposed to 900 and 2100 MHz RFR for 2 h/day. There were 20 male Wistar rats in each group. Immunohistochemistry stainings were performed GRP78, CHOP, Cleaved (Clv.) Caspase 3 and Caspase 12.

Results: Expressions of GRP78 and Clv. Caspase3 in RFR groups is significantly higher than sham groups ($p < 0.001$). In 900 MHz-1 w rats, high levels of GRP78 expressions were at the cytoplasm of epithelial reticular cells. In other groups, GRP78 expressions were seen also at thymocytes. Expressions of CHOP in RFR rats were higher than sham rats (2100 MHz /Sham for 10 w; $p < 0.001$, 900 MHz /Sham for 10 w; $p = 0.004$, 900 MHz /Sham for 1 w; $p = 0.003$). Localization of CHOP expressions was at the nucleus membrane and cytoplasm. The expression of Caspase 12 in RFR rats was higher than sham rats (900 MHz /Sham for 1 w; $p = 0.006$, other groups; $p < 0.001$).

Conclusions: This study demonstrates RFR exposure could increase levels of ER stress pathway proteins and could cause apoptosis.

Keywords: Radiofrequency radiation, thymus, endoplasmic reticulum stress, apoptosis

The thymus is a primary lymphoid organ located behind the sternum, in the superior mediastinum. The thymus has an important role in the selection and maturation of T lymphocytes.

Endoplasmic reticulum (ER) is the biggest or-

ganelle in the cell. ER's essential functions are lipid synthesis, protein folding, and synthesis, Ca⁺⁺ homeostasis, detoxification. Hypoxia, cancer, reactive oxidants, low Ca⁺⁺ levels, and many other conditions can disrupt these functions [1]. Thus, unfolded, and dys-

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functional proteins accumulate in the ER and cause ER stress. ER stress initiates Unfolded Protein Response (UPR) [1, 2]. UPR tries to solve this ER stress problem in various ways. For instance, to increase ER protein turnover, to activate up-regulation of chaperons, etc... If the cell can not overcome ER stress, UPR initiates cell death programs [2].

UPR is controlled by three transmembrane sensors: PERK (protein kinase RNA-like endoplasmic reticulum kinase), IRE-1 (Inositol regulating enzyme-1) and ATF-6 (Activating Transcription Factor-6). Sensors detect unfolded proteins and activate the other steps [1, 2]. In normal conditions, a chaperon and calcium-binding protein; 78-kDa glucose-regulated protein (GRP78, or the other name Binding immunoglobulin protein-BiP) stabilizes the inactive state of sensors. But unfolded proteins send away GRP78, so UPR sensors become free and active [3].

Active PERK inhibits protein synthesis by phosphorylating eIF2 α . PERK induces translation of some proteins, the most researched one is activating transcription factor 4(ATF4). ATF4 induces chaperon synthesis, antioxidant synthesis, C/EBP homologous protein (CHOP) synthesis, and autophagy genes. CHOP regulates BCL-2 family genes which are responsible for apoptosis. CHOP also induces proapoptotic genes and reduces antiapoptotic genes [1, 4]. PERK pathway also plays role in mitochondrial pro-survival signaling, mitochondrial autophagy, and mTOR-P3IK-Akt pathway [4].

IRE1 is an endoribonuclease localized in the ER membrane. If ER stress is present, IRE1 splice x-box binding 1 protein (XBP1) mRNA. XBP-1 induces UPR genes. Persistent IRE1 activation could trigger apop-

toxis via tumor necrosis factor receptor-associated factor (TRAF2) [5]. ATF6 induces XBP1 synthesis and chaperon synthesis like GRP78. Also, ATF6 induces genes that play a role in the degradation of misfolded proteins [6].

RFR is a power caused by the energy of electrical charges. It was classified by European Committee (Table1) [7]. As seen in the table mobile phones, tablets, electrical appliances, and others are radiofrequency radiation (RFR) sources. We are exposed to RFR intensely these days. Therefore, it is important to understand how RFR affects the organism. Some researchers have investigated RFR's effects on the thymus. In research published in 2011, it was demonstrated that 900MHz RFR increases superoxide dismutase (SOD) levels, glutathione peroxidase (GPx) activity, and decreases glutathione levels in rat thymus [8]. Hanci *et al.* [9] reported that exposure to 900 MHz RFR caused an increase in malondialdehyde (MDA)-an oxidative stress marker- and decreased glutathione levels in prenatal rat thymus. Also, it was reported that RFR could lead to pathological changes in prenatal rat thymus [9]. Another research showed 900 MHz RFR causes an increase in MDA levels and induces histopathological changes at mature rat thymus [10, 11]. Misa-Agustino *et al.* [12]. have applied 2.45 GHz (0-1-1.5-12-Watt power) RFR to rats. In this study heat shock protein (HSP) 90 decreased in only 12 W exposed animals and there were no differences in HSP70 levels. In exposed rats, glucocorticoid receptors presented more than control groups.

As shown in the studies, exposure to RFR could induce oxidative stress and make changes at thymus histopathology. There is no study about RFR's effects

Table 1. Electromagnetic fields and sources

Frequency Range	Frequencies	Some examples of exposure sources
Static	0 Hz	VDU (video display); MRI and other diagnostic/scientific instrumentation; Industrial electrolysis; Welding devices
ELF	0-300 Hz	Powerlines; Domestic distribution lines, Domestic appliances; Electric engines in cars, train and tramway; Welding devices
IF	300 Hz- 100 kHz	VDU; anti theft devices in shops, hands free access control systems, card readers and metal detectors; MRI; Welding devices
RF	100 kHz – 300 GHz	Mobile telephony; Broadcasting and TV; Microwave oven; Radar, portable and station radio transceivers, personal mobile radio; MRI

VDU = video display units for computers, videos, television using cathode ray tubes, ELF = extremely low frequency, IF = intermediate frequency, RF = radio frequency, MRI = magnetic resonance imaging, TV = television

on ER stress in the thymus. We investigate in this study that 900 and 2100 MHz RFR whether could cause ER stress in rat thymus.

METHODS

Animals and Study Design

This study was conducted on 120 male Wistar rats aged 3 months and weighing 250-300 gr. All animals were kept in a controlled environment with the artificial light-dark cycle of 12 h lights on and 12 h lights off and received food and tap water ad libitum. There were 4 animals in every cage. Sham rats were housed in separate rooms under the same conditions for an equal time. All experimenters were blind to animal experimental group memberships during the data collection and analysis. The animals were randomly divided into 6 groups like below.

Groups:

1. Sham 1w (acute)
2. Sham 10w (chronic)
3. Exposure to 900 MHz RFR for 1w (acute)
4. Exposure to 900 MHz RFR for 10w (chronic)
5. Exposure to 2100 MHz RFR for 1w (acute)
6. Exposure to 2100 MHz RFR for 10w (chronic)

Ethical Approval

Ethical approval for this work was obtained from Akdeniz University Local Committee on Animal Research Ethics (2013.05.01). All experimental protocols

conducted on rats were performed under the standards established by the Institutional Animal Care and Use Committee of Akdeniz University.

Radio Frequency Radiation Exposure

The radio frequency (RF) values used in this study are the frequencies (900 and 2100 MHz) used in mobile phone communication. An RF generator (GSM Simulator; Everest Company, Adapazari, Turkey), which produces 900 and 2100 MHz RF radiations, was used to represent exposures of global systems for mobile communications (GSM). In the 900 MHz RFR experiment the carrier frequency was 900 MHz, the modulation frequency was 217 Hz, the pulse width was 0.577 msec, and the power range of the generator was 0-10 W. Besides in the 2100 MHz RFR experiment, the carrier frequency was 2100 MHz, the modulation frequency and the pulse width were the same as 900 MHz and the power range of the generator was 0-2 W.

The animals were placed in plexiglass tubes with holes where one single rat could fit, breathe comfortably, and not increase the body temperature. Rats in plexiglass tubes were placed radially at equal distances (in 900 and 2100 MHz experiments rats' noses were at 3.5 and 10 cm distances from the antenna, respectively) around the antenna (Fig.1). The applied carousel setup procedure of the present study was by the setup procedure of the other studies in the literature [13-15]. The tubes restrained the movement of the rats to such an extent as to follow for well-defined exposure conditions, yet without immobilizing them. RFR

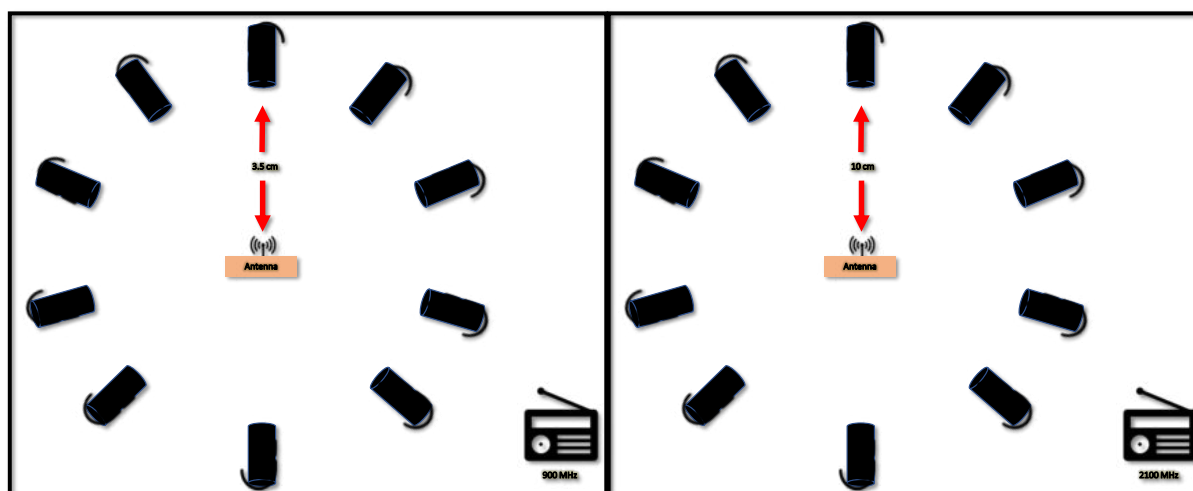


Fig. 1. The experimental setup for the exposure of 900 and 2100 MHz RFR.

application was held in a shielded room to prevent the rats from the effects of other electrical sources. For the used frequencies, the source output powers were selected by considering the power values emitted by the mobile phones (the output powers adjusted to 5 W (for 900 MHz) and 1.5 W (for 2100 MHz) during the exposure). During the experiment in the 'signal on' position, the measured electric-field strengths over the rat's heads were 35.5 and 35.2 V/m for 900 and 2100 MHz frequencies, respectively.

Group 3 rats were exposed to 900 MHz RFR 2 hours/day, 5 days/week for 1 week, and group 4 rats were exposed to the same frequency RFR 2 hours/day, 5 days/week for 10 weeks. Group 5 rats were exposed to 2100 MHz RFR for the same duration as group 3 and group 6 rats were exposed to 2100 MHz RFR for the same duration as group 4. Sham rats were kept under the same conditions and durations while the RFR generator was turned off for 1 week and 10 weeks.

Electric field strengths were measured by EMR300 (Narda, Germany) with a suitable probe in the experiments. The electric field background level was between 0.02 and 0.2 V/m in the shielded room. Also background magnetic fields were measured between 0.01 and 0.03 μ T by Hioki 3470 Magnetic Field Hitester (Hioki E. E. Corp., Japan) with an appropriate probe.

Dosimetry simulations were carried out using a finite integration technique (FIT) based commercial software, CST Microwave Studio (3D EXPERIENCE®, Dassault Systemes, Hamburg). The FIT was introduced by Weiland [16]. Although the gridding can be applied as a finite difference time domain (FDTD) method, the FIT uses the integral form of Maxwell's equations [17]. In the present study, the rat model used in simulations has consisted of voxels with a resolution of $1.827 \times 1.827 \times 2.015$ mm³. The interaction between the incident electromagnetic wave and the biological tissue are explained by electrical properties that can be obtained by the dielectric properties of the interested tissue [18, 19]. In this study, each tissue of the simulated rats has its electrical properties at the operating frequencies. The average whole-body SAR values at 900 and 2100 MHz were 1.159 and 0.16 W/kg, respectively. Besides the SAR values for the thymus at 900 and 2100 MHz were on the average of 1.134 and 0.086 W/kg, respectively. Before and after

all experimental sessions, the body temperatures of rats were monitored by rectal measurements. The RFR exposure did not lead to any rectal temperature raise.

Tissue Sample Obtaining and Processing

Thymus tissues were fixed by 10% formaldehyde solution. Tissues were processed through graded alcohols and xylene, then embedded in paraffin. Sections were cut 5-7 μ m using a rotary microtome (Leica, Nussloch, Germany) and taken on Superfrost slides.

Immunohistochemistry

Prepared sections were stained immunohistochemically for GRP78, CHOP, Caspase 12, and Clv. Caspase3 proteins. Protein localization and levels of expressions were evaluated.

Thymus tissue samples were deparaffinized in xylene and dehydrated in ethanol series. Slides were boiled at 665 W microwave and waited 25 minutes at microwave's warm position, then kept at room temperature (RT) for 20 minutes in Tris-EDTA solution (pH = 9.0). They were incubated in 3% hydrogen peroxide (Sigma Aldrich 18312), made with methanol, to block endogen peroxidase activity and washed with phosphate-buffer-saline (PBS). Then nonspecific binding sites were blocked with UV Blocking Solution (#TA-125-UB; Thermo Scientific/Lab Vision). Primary antibodies, prepared with an antibody diluent solution, were incubated for one night at +4°C GRP78 1/400 (ab21685; Abcam), CHOP :1/50 (ab11419; Abcam), Caspase 12 1/50 (ab62463; Abcam), and Clv. Caspase3 1/50 (9664; Cell Signaling). After washing, secondary antibodies were incubated for 45 minutes at RT. They have washed again and incubated with streptavidin (TS-125-HR; Thermo Scientific/Lab Vision) for 30 minutes at RT. After washing, antibodies were identified with DAB(8050S; Cell Signalling) and then again washed. After Mayer's hematoxylin staining (Merck) and dehydration, mounting was done with Entellan (#1.07961.0100; Merck). The immunolocalization of the ER stress proteins was examined with bright-field microscopy (Zeiss). We have analyzed expression levels of proteins by using ImageJ software.

Statistical Analysis

Immunohistochemical stainings were analyzed by using Image J. The data were evaluated by using

Sigma Stat 3.5 One Way ANOVA/post hoc Tukey test. Values with $p < 0.05$ were considered statistically significant. The results were presented as mean \pm SEM.

RESULTS

GRP78, an important protein of the UPR, increased in 900MHz-1 w relative to the Sham-1 w. But this increase was just in the cytoplasm of epithelial reticular cells. This increase was both at epithelial reticular cells and thymocytes in the 900MHz-10 w, 2100MHz-1 w,

and 10 w groups (Fig. 2). Exposure to RFR can induce ER stress in thymocytes which have an important role in the immune system. Additionally, exposure duration of RFR and frequency of RFR can change this effect (Fig. 3).

If cells cannot handle ER stress, CHOP levels will increase, and CHOP induces proapoptotic genes and by the way apoptosis. In 10-w groups, an increase of CHOP expression was observed, and this increase was in the nuclear membrane and cytoplasm. Caspase12 showed a significant increase depending on RFR exposure and frequency levels. CHOP and Caspase12

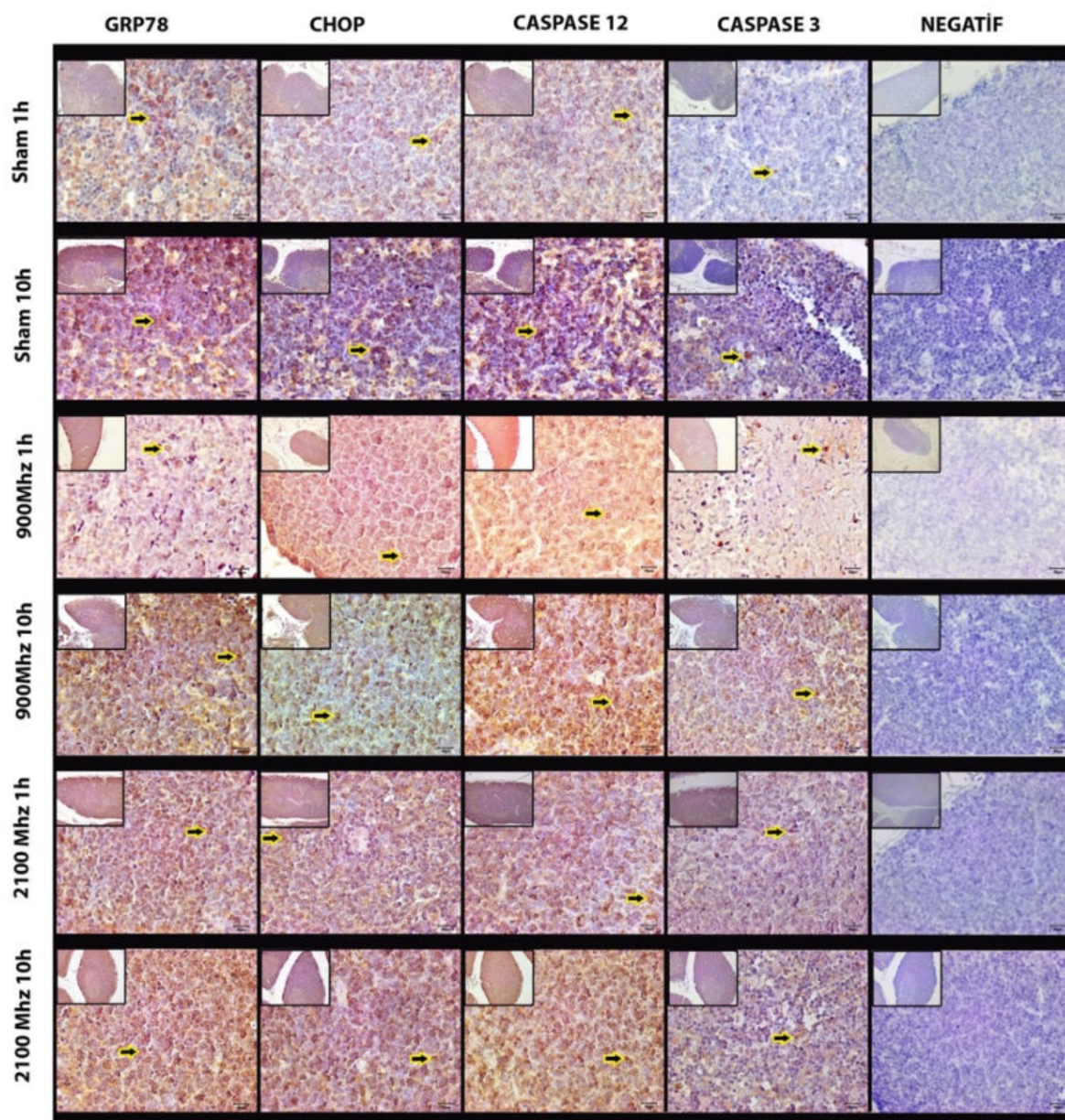


Fig. 2. Hematoxylin staining for all groups and immunohistochemistry staining for GRP78, CHOP, Clv. Caspase3 and Caspase12,10 \times and 40 \times , bar 50 μ m.

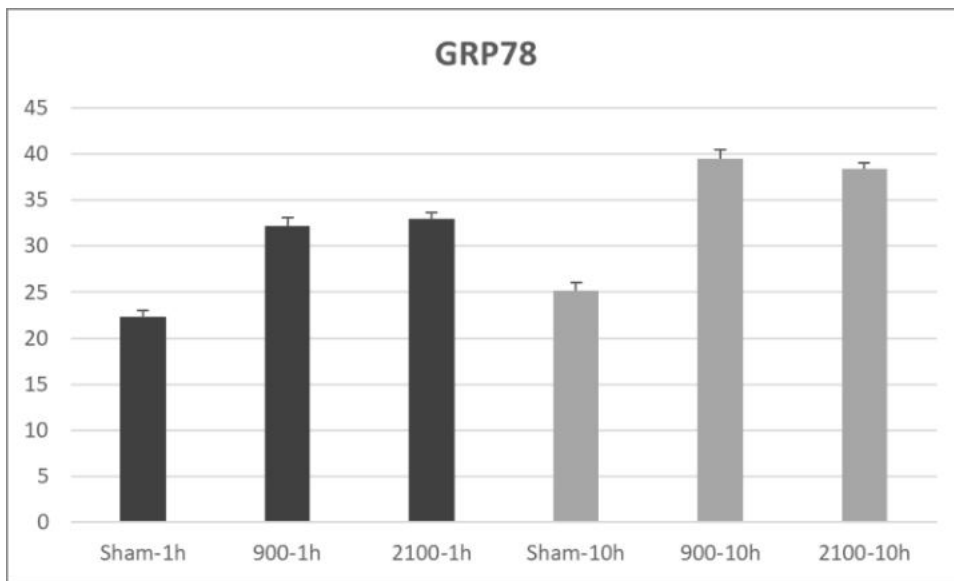


Fig. 3. ImageJ analyze for the immunohistochemistry staining GRP78. 900 MHz 10 w vs. Sham 1 w, 900 MHz 10 w vs. Sham 10 w, 900 MHz 10 w vs. 900 MHz 1 w, 900 MHz 10 w vs. 2100 1 w, 2100 MHz 10 h vs. Sham 1 w, 2100 MHz 10 h vs. Sham 10 w, 2100 MHz 10 w vs. 900 MHz 1 w, 2100 MHz 10 w vs. 2100 1 w, 2100 1 w vs. Sham 1 w, 2100 MHz 1 w vs. Sham 10 w, 900 MHz 1 w vs. Sham 1 w and 900 MHz 1 w vs. Sham 10 w ($p < 0.001$).

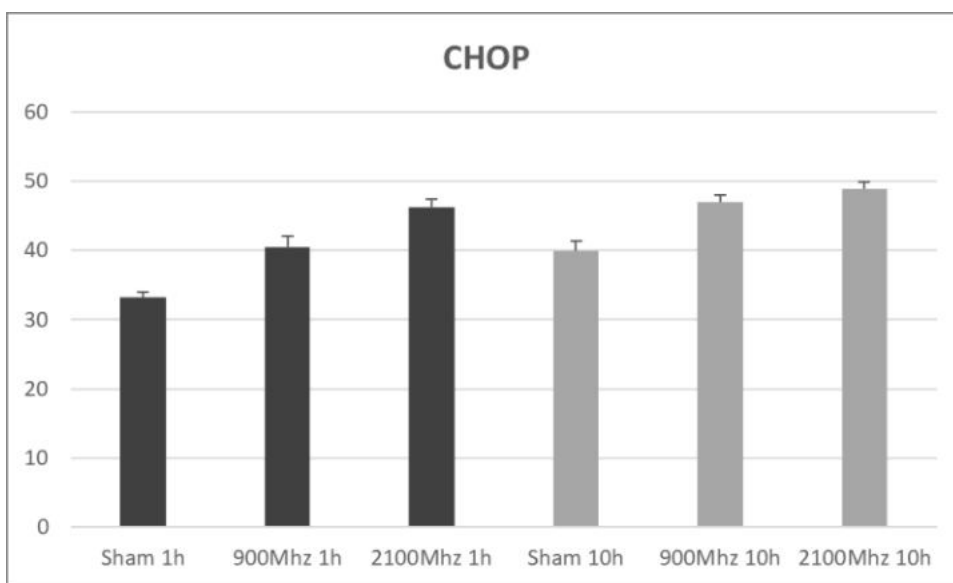


Fig. 4. ImageJ analyze for the immunohistochemistry staining CHOP. 10 w vs. Sham 1 w, 2100 MHz 10 w vs. Sham 10 w, 2100 MHz 10 w vs. 900 MHz 1w, 900 Mhz 10 w vs. Sham 1 w and 2100 MHz 1w vs. Sham 1 w ($p < 0.001$), 900 MHz 10 w vs. Sham 10 w ($p = 0.004$), 900 MHz 10 w vs. 900 MHz 1 w ($p = 0.008$), 900 MHz 10 w vs. 2100 MHz 1 w ($p = 0.999$), 2100 MHz 1 w vs. Sham 10 w ($p = 0.01$), 2100 MHz 1 w vs. 900 MHz 1 w ($p = 0.02$), 900 MHz 1 w vs. Sham 1 w ($p = 0.003$), Sham 10 w vs. Sham 1 w ($p = 0.007$).

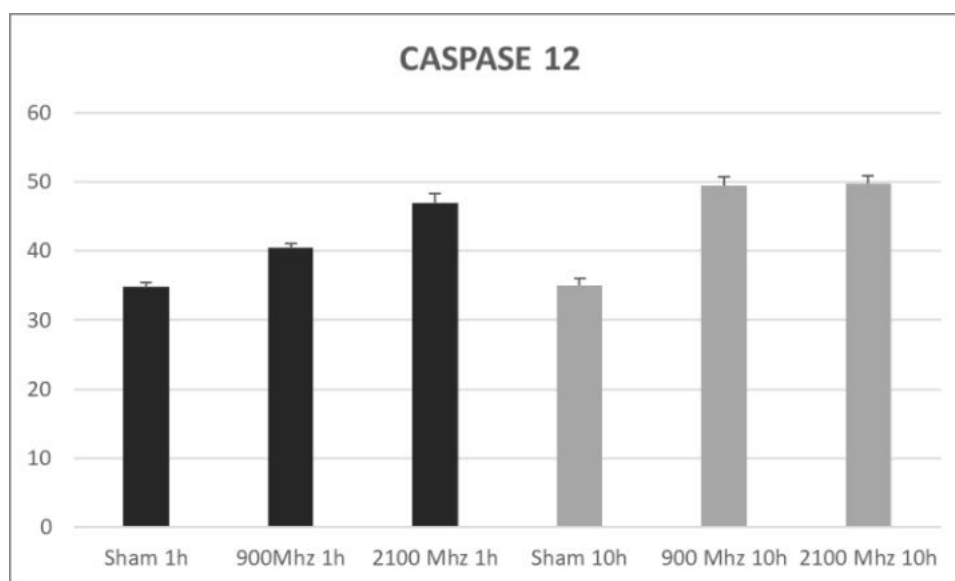


Fig. 5. ImageJ analyze for the immunohistochemistry staining Caspase 12. 2100 MHz 10 w vs. Sham 1 w, 2100 MHz 10 w vs. Sham 10 w, 2100 MHz 10 w vs. 900 MHz 1 w, 900 MHz 10 w vs. Sham 1 w, 900 MHz 10 w vs. Sham 10 w, 900 MHz 10 w vs. 900 MHz 1 w, 2100 MHz 1 w vs. Sham 1 w and 2100 MHz 1 w vs. Sham 10 w ($p < 0.001$), 2100 MHz 1 w vs. 900 MHz 1 w ($p = 0.001$), 900 MHz 1 w vs. Sham 1 w ($p = 0.006$), 900 MHz 1 w vs. Sham 10 w ($p = 0.009$).

levels were also high in sham groups, but the increases in RFR groups were significantly higher (Figs 2, 4 and 5).

Clv. Caspase 3 levels were higher in RFR groups than sham groups. But the change of frequency did not affect meaningfully Clv. Caspase 3 levels. Interestingly, the expression of Clv. Caspase 3 in 1w RFR groups were higher than 10w RFR groups (Fig. 6). These three markers were observed in epithelial reticular cells and thymocytes (Fig. 2).

DISCUSSION

With the developing technology, RFR has become an important part of human life. Unfortunately, the effects of RFR on ER stress and apoptosis have not been clarified yet. There are too many studies about the effects of RFR, in different frequency levels, on the organism. We will discuss here only the studies about exposure to 900 MHz and 1950-2450 MHz RFR. We have investigated the effects of RFR on ER stress and apoptosis in rat thymus in our study.

As known oxidative stress is a possible trigger of ER stress and apoptosis. There are many studies on

different tissues about this subject. In a study on the sciatic nerve of adolescent rats, 900MHz RFR increased MDA, SOD, and catalase (CAT) levels and also, induced apoptosis [11]. 1950 MHz RFR exposure for 48h, not 12 or 24 h, could induce apoptosis on rat astrocytes according to Liu *et al.* [20]. In another study, no difference was detected between 900 MHz 0 h and 24 h groups of rats [21]. As can be seen, exposure duration is important for the effects of RFR. There are also several studies in the urogenital system. There are studies for male or female rats, prenatal or natal exposure, different exposure durations, and frequencies. These studies [22-33] demonstrated that RFR could induce oxidative stress and apoptosis in testis and kidney tissues. Further in another study, 900 MHz RFR increased oxidative stress in the ovary [34]. In different studies on the prenatal and adolescent rats, exposure to 900 MHz RFR caused oxidative stress [35, 36]. Exposure to 2450 MHz RFR caused oxidative stress to the heart, but there was no difference in the blood tissue [37].

As for the immune system, studies on the liver suggested that RFR could induce oxidative stress and decrease antioxidant levels in the rat liver [38-40]. Ohtani et al. [41]. exposed the rats to 2.14GHz RFR

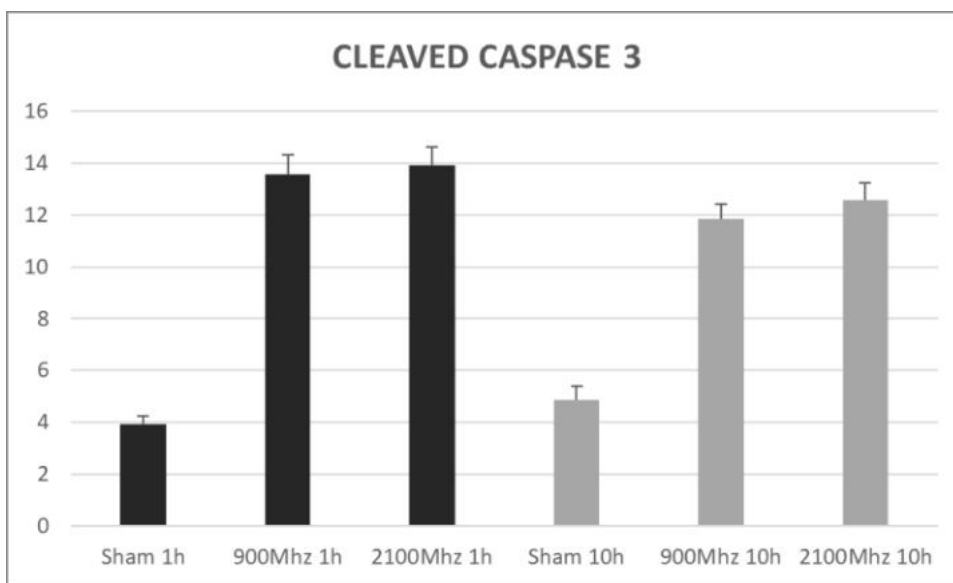


Fig. 6. ImageJ analyze for the immunohistochemistry staining Clv. Caspase 3. 2100 MHz 1 w vs. Sham 1 w, 2100 MHz 1 w vs. Sham 10 w, 900 MHz 1 w vs. Sham 1 w, 900 MHz 1 w vs. Sham 10 w, 2100 MHz 10 w vs. Sham 1 w, 2100 MHz 10 w vs. Sham 10 w, 900 MHz 10 w vs. Sham 1 w and 900 MHz 10 w vs. Sham 10 w ($p < 0.001$).

for 9 weeks which was included in utero, lactation, and juvenile period. They presented IL4-IL5 and IL23a genes in the thymus and just IL5 gene in spleen up-regulated. But there was no effect on the growth of T cells [38, 41]. In another study, rats were exposed to 900 MHz RFR during the prenatal period. In this study increased MDA levels, decreased GSH levels, and not changed SOD levels were found in the exposed thymus. Also for the spleen, increased MDA and GSH, but decreased SOD levels were found [9]. For the natal exposure in the thymus, researchers found high MDA levels in exposed groups (900MHz) [10]. Exposure to 900 MHz RFR in mature or immature rats causes increased SOD levels, decreased CAT-GPx activity, and GSH levels in all lymphoid organs according to Aydin et al. [8]. Misa-Agustino et al. [12]. applied 2.45 GHz (0-1-1.5-12-Watt power) RFR to rats and studied on thymuses. In this study heat shock protein (HSP)90 decreased in only 12W exposed animals and there were no differences in HSP70 levels. Also, in exposed rats, glucocorticoid receptors presented more than control groups [12].

In the mentioned studies, researchers used 900 MHz or 1950-2100-2450 MHz RFR like our study. The exposure durations and frequencies were different in these studies and findings could be different due to

this. As shown, exposure to 900-2100 MHz RFR, prenatal or natal, can cause oxidative stress and apoptosis in different tissues. But in some studies, it was found that there is no specific effect of RFR [21, 38, 39]. In our study, rats were exposed to RFR at least 1w (2h/day). Caspase12 and Clv. Caspase3 were evaluated for apoptosis, GRP78 and CHOP were evaluated for the oxidative stress relevant to ER stress. Exposure to 900-2100 MHz RFR for 1 w or 10 w increased CHOP and GRP78 expressions in the thymus (for CHOP: 2100/Sham 10 w; $p < 0.001$, 900/Sham 10 w; $p = 0.004$, 900/Sham 1 w; $p = 0.003$ and for GRP78; $p < 0.001$). Also, these exposures increased Clv. Caspase 3 and Caspase 12 expressions (Caspase 12: 900 MHz 1 w/Sham 1 w; $p = 0.006$, 900 MHz 10 w/Sham 10 w, $p = 0.01$, 2100 Mhz/Sham; $p < 0.001$ and cl Caspase 3; $p < 0.001$).

CONCLUSION

We demonstrated that exposure to RFR -acute or chronic, 900 MHz or 2100 MHz- causes ER stress and apoptotic markers in rat thymus. These findings are compatible with the literature. The effects of RFR on the thymic cells of rats, one important part of the im-

mune system, suggest that RFR may cause various pathological diseases and weaken our immunity via cell death.

Authors' Contribution

Study Conception: ErK, EsK; Study Design: ErK, EsK, HE, AOO, ŞÖ, ND; Supervision: EsK, ND; Funding: TUBITAK; Materials: ErK, EsK, HE, AOO, ŞÖ, ND; Data Collection and/or Processing: ErK, EsK, HE, AOO, ŞÖ, ND; Statistical Analysis and/or Data Interpretation: ErK, EsK; Literature Review: ErK, EsK; Manuscript Preparation: ErK, EsK and Critical Review: ErK, EsK.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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