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Effects of pre and postnatal 2450 MHz continuous wave (CW) radiofrequency radiation on thymus: Four generation exposure

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ABSTRACT

This study aims to investigate the effects of pre- and postnatal 2450 MHz continuous wave (CW) radiofrequency radiation (RFR) on the thymus of rats spanning four generations. Four groups; sham, irradiated female, irradiated male, irradiated male and female, each consisting of four rats (one male and three females), were created. During the experiment, rats in the exposure groups were whole-body exposed to 2450 MHz CW-RFR for 12 h/day. Irradiation started one month before the fertilization in the experimental group. When the offspring were two months old, four rats, one male and three female, were allocated for the second-generation study. The remaining offspring were sacrificed under general anesthesia, and their thymuses were removed. The same procedure was applied to the next generation. Two months after the second generation gave birth, third-generation rats were decapitated, and their thymuses were removed. In all groups, cortex, medulla and resident cells could be clearly distinguished in the second and third generations. No differences were observed between the control and two experimental groups, defined as irradiated female and irradiated male. In contrast, vascularization was observed in the thymus of the fourth-generation offspring of the group where both males and females were irradiated. The number of offspring and mass of all rats decreased in the third-generation group. Pre- and postnatal 2450 MHz continuous wave radiofrequency radiation exposure may potentially affect the thymus of future generations.

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KEYWORDS

2450 MHz RF; pre- and postnatal exposure; continuous wave; thymus; four generations

Introduction

The classification of radio frequencies (RF) as 2B agents by the International Agency for Research on Cancer (IARC) and the common use of wireless communication (Wi-Fi) devices in data transmission have raised health fears associated with electromagnetic fields (EMF) exposure. Developing tissues and organs are susceptible to harmful agents. The World Health Organization (WHO) has led research on the effect of early-life exposure to microwave (MW) as high precedence for 2006 and 2010 research on EMF because of the possible vulnerability of children (Laudisi et al. 2012).

Long-term pre- and postnatal RF radiation (RFR) exposure and the delayed effects are not known sufficiently. As the effects of RFR are still uncertain, the Council of Europe recommends that restrictions be placed on internet access and usage of mobile phones in all schools to preserve juveniles from the potential harm of radiation (Watson 2011). There are many reports about the effect of mobile phone exposure on

health, but research on the effect of Wi-Fi 2.4 GHz RFR is considerably less than mobile phones (Dasdag et al. 1999, 2000, 2009; Dasdag et al. 2015a). However, Dasdag et al. investigated the effect of pulsed 2.4 GHz RFR on the brain and found it can cause adverse effects (Dasdag et al. 2015b). Although many organs, especially the brain, have been the focus of these studies, the thymus is often overlooked and has not been considered as being affected by RFR.

The thymus is a central organ of the immune system and the lymphatic system, which differentiate into antigen-recognizing cells and lymphocytes produced by the bone marrow in mammalian cells (Dominguez-Gerpe and Rey-Menéndez 2003; Miller 2020; Rezzani et al. 2014). During early embryogenesis, with the onset of hematopoiesis begins development of the immune system and then throughout prenatal life continues with successive hematopoietic cell production, cell migration, and differentiation (Laudisi et al. 2012). The thymus is vulnerable to physiological alters such as pregnancy,

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aging, and external factors. In recent decades, researchers have found that ionizing and non-ionizing radiation achieve an external stimulus that changes thymus functions and modulates immune responses (Chen et al. 2000; Hekmat et al. 2013; Quaglini et al. 2004).

It was reported that CD8 + T and mature CD4+ cells migrated from the thymus into peripheral blood, initiating immune surveillance. The thymus was highly radio-sensitive because of the rapid proliferation and turnover of thymocytes (Wu et al. 2020). Wu et al. also stated that “Radiation damage was found to be characterized by important tissue-specific restoration and complexity patterns, especially in the thymus.” Sambucci et al. investigated the thymocytes maturation effects of Wi-Fi signals. The early life exposure to 2.45 GHz Wi-Fi 33-like signals showed no differences in total cell number however it showed differences between the thymuses of exposed mice. Therefore, they reported that their results did not exhibit on thymocyte development any effects of early life exposure to Wi-Fi signals (Sambucci et al. 2011). Furthermore, Laudisi et al. investigated the thymocyte development and T cell effects of prenatal Wi-Fi signals. They stated that prenatal Wi-Fi exposure did not cause detrimental effects on T cell development (Laudisi et al. 2012). Most of the studies discussed above examined the effects of prenatal irradiation on the fetus. Therefore, studies in which prenatal irradiations were performed mainly were based on whether the babies born were affected or not. Furthermore, limited studies have addressed the effects of pulsed RFRs, and the effects of the continuous (CW) RFR are unknown. The effects of irradiation spanning several generations have not been examined to date. The purpose of this study is to investigate the effects of pre-and postnatal 2450 MHz CWRFR on the thymus of consecutive generation rats.

Materials and methods

The experimental animals

All provisional protocols applied to rats were carried out in accordance with the Principles of Laboratory Animal Care and the rules of the Scientific and Ethics Committee of Experimental and Clinical Research Center (DEKAM) of Erciyes University. This research has been approved by the Local Ethics Committee of Animal Experiments of Erciyes University (Decision No: 16/111). Twelve female and four male Wistar Albino rats were used in this study at the beginning. All rats were acquired from Erciyes University Experimental Research and Application Center (Kayseri, Turkey). Rats were housed under standard conditions (12 h light/12 h dark cycle and the temperature kept at

$23 \pm 2.5^{\circ}\text{C}$). Rats were fed ad libitum, and tap water was provided as drinking water. Water and food were available from the center.

The rats were divided into sham control and three exposure groups, with one male and three female rats in each group (Figure 1). During the experiment, rats in the exposure groups were whole-body exposed to 2450 MHz CW-RFR for 12 h/day. In the first exposure group, only male rats were exposed. Both female and male rats were exposed in the second exposure group, and in the third group, only female rats were exposed. The same process was applied to rats in the sham control group by turning off the generator.

Radiofrequency radiation exposure

An RFR generator (220 V; 2370–2450 MHz, 0–4 Watt; Nar Technology, Kayseri, Turkey) with 2450 MHz CW utility was used in the study. The study was performed in a Faraday cage to eliminate environmental exposures from other RF devices such as mobile phones, base stations, radio-TV transmitting stations. A cylindrical cage with wire grid top and bottom covers enables free-air access to the rats during exposure measurements. Wire-grid spacing was selected as 5 mm, which was much smaller than free-space wavelength λ_0 , at 2.45 GHz. Experimental and simulation setups are illustrated in Figure 2. Before RFR exposure, ambient RF level was measured with a wideband antenna (Log-periodic Antenna, HF59B, FF6E, Gigahertz Solutions, Langenzenn, Germany) in the inner and outer surface of the cylindrical cage. The power of the RF generator was set to 1 Watt. RF field levels inside and outside the cylindrical cage was remeasured with the generator turned on. The electromagnetic power density was recorded as $8.5 \pm 0.65 \text{ W/m}^2$ inside each section of the cylindrical cage. Rats could freely move in the cage. The entire study was conducted at the Erciyes University Research Center in a room (Temperature: $25 \pm 2^{\circ}\text{C}$, Humidity: 40%-50%, 12 h constant light-dark cycle) specifically allocated for this research. Animals were fed ad libitum, and tap water was provided as drinking water. All the animals were exposed to RFR between 08:00 and 20:00 during the study.

RF exposure started one month before the fertilization, and the fertilization took place at the end of this period (first-generation). The pregnancy was determined by a vaginal smear test. When the offspring were two months old (second-generation), four rats, one male and three female, were allocated for the next generation study. The remaining offspring were sacrificed under general anesthesia, and their thymuses were removed. After the second generation gave birth, the

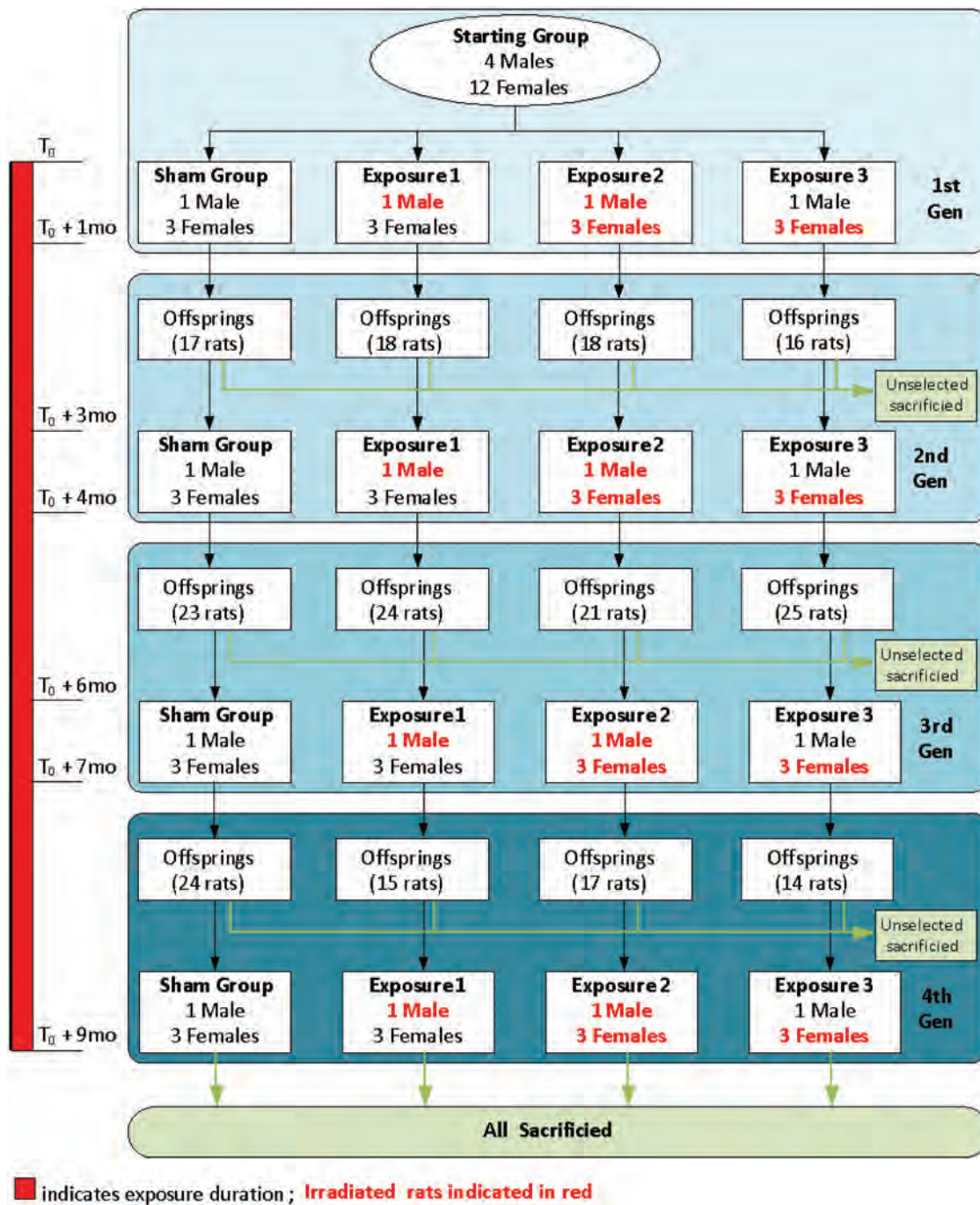


Figure 1. Rats in respective groups and generations.

offspring (third-generation) were grown for two months, and then, all rats were decapitated under general anesthesia to remove their thymus. After the third generation gave birth, the offspring (fourth-generation) again were let grow for two months, and then, again, all rats were sacrificed under general anesthesia to extract their thymuses. In summary, the irradiation started one month before the pregnancy of the first generation and continued until the fourth-generation animals were

sacrificed. However, successive generation animals were also designed and experienced the same processes as the first-generation rats (Figure 1).

Specific absorption rate (SAR)

The exposure setup with the antenna, the Faraday cage, and the rats was modeled in a 3D electromagnetic field solver using CST (CST AG, Darmstadt,

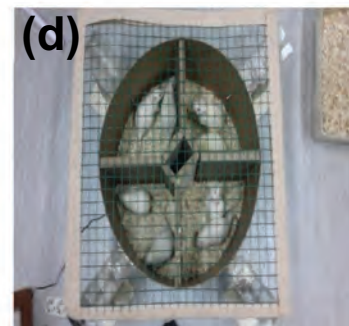
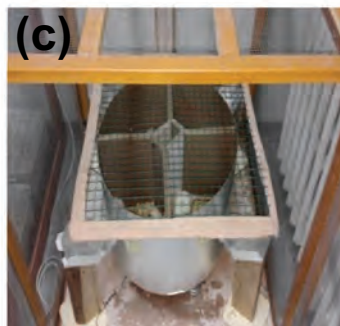
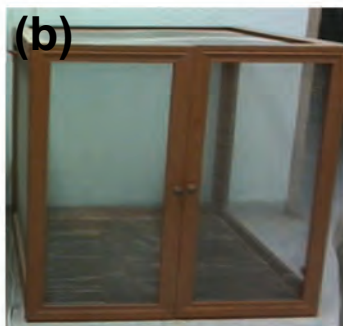
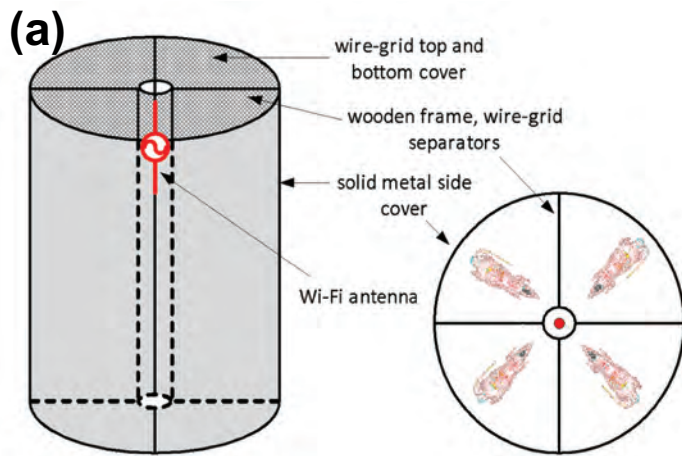


Figure 2. Simulation and experimental setups. (a) Cylindrical cage for RF irradiation, (b) large cylindrical cage to house rats, (c) The cylindrical cage inside the rectangular Faraday cage, (d) top view of the Faraday cage.

Germany). CST is based on the finite-integration technique, which employs discretization in the integral form of electromagnetic fields rather than their derivatives. Whole-body SAR's were calculated as 0.186 W/kg for 1 g average and 0.093 W/kg for 10 g average using IEEE/IEC 62704-1 method. SAR at thymus was calculated as 0.051 W/kg and 0.068 W/kg for 10 g and 1 g averaging, respectively. Rat voxel model and sagittal plane SAR distribution for 1 g averaging are displayed in Figure 3(a, b), respectively. Since the antenna and the rats were symmetrically located in the simulation setup, no variation of SAR from one chamber to another was observed. However, dosimetry levels are expected to change slightly due to size differences between the animals. This variation is expected to be small as the mass of rats does not differ more than 5% of the mean mass at exposure groups. Another 3D electromagnetic field simulator, Sim4Life (ZMT Zurich MedTech AG, Zurich, Switzerland), was used to simulate SAR for offspring because only Sim4Life had a 7-day old infant rat voxel model (Figure 3c),

which could be used for SAR simulations. Sim4Life utilizes finite difference time domain technique as 3D electromagnetic fields solver. The same exposure setup was replicated in Sim4Life, and SAR at thymus was calculated as 0.116 W/kg for 1 g averaging. Whole-body SAR for offspring was calculated as 0.287 W/kg. For offspring, whole-body SAR and at the thymus were 1.54 times and almost twice more than adults, respectively.

Histopathological evaluation

The thymus morphology was evaluated hematoxylin-eosin staining in two-month-old rats. After, thymus tissues were excised from each animal, and the tissues were cut longitudinally. The tissues were fixed in 10% formaldehyde for 24 h. Specimens were dehydrated through graded concentrations of ethanol, cleared with xylol, and embedded in paraffin wax. 5 μ m thick slices were cut from each paraffin block and stained with hematoxylin and eosin to show histological changes.

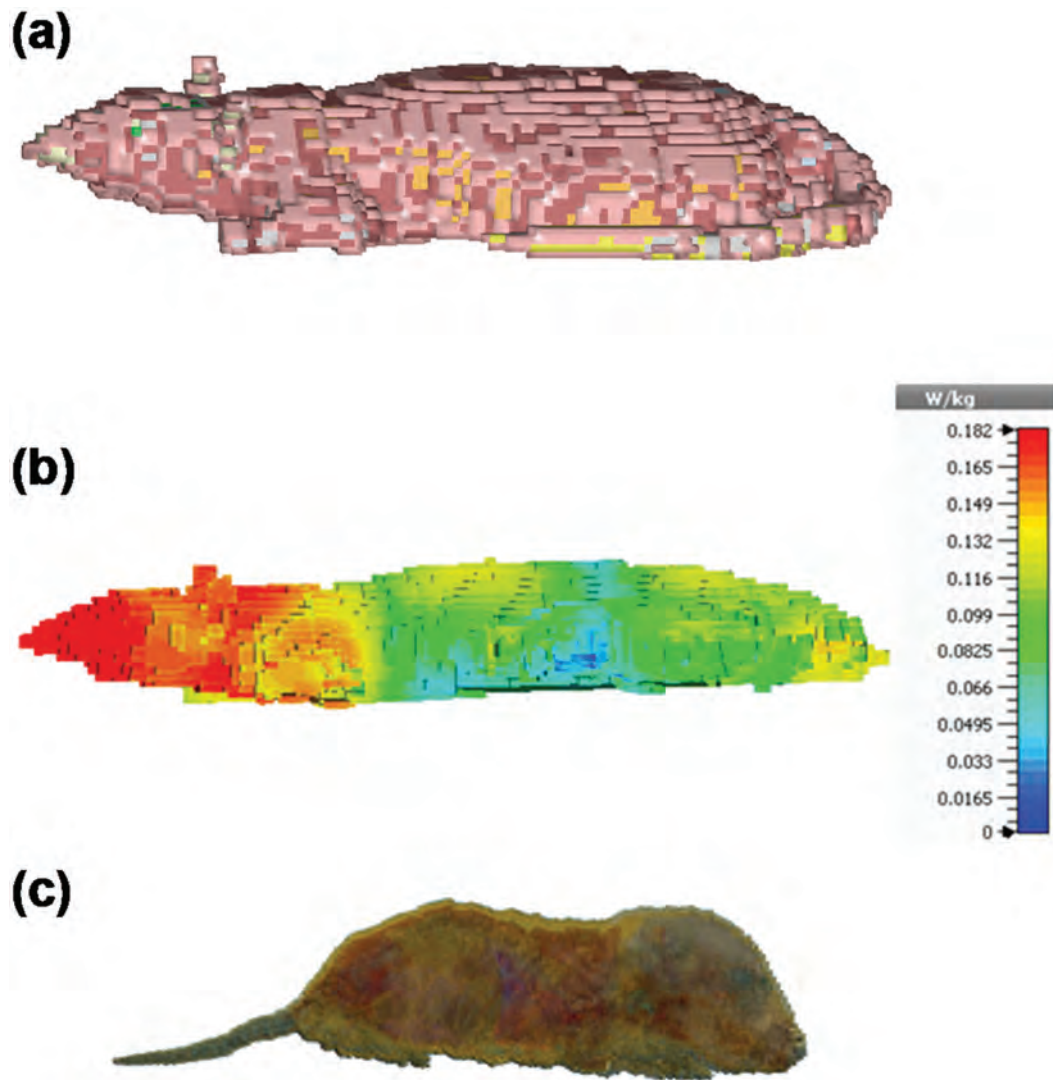


Figure 3. SAR simulations at 2450 MHz. (a) Adult rat voxel model, (b) adult rat sagittal cut whole body SAR distribution for 1 g average, (c) one week old baby rat model.

After staining, the slices were examined using a light microscope (Olympus BX51, Tokyo, Japan) and images were captured.

Results

Differences were not recorded in the gross anatomy of all generation rats. However, visibly pronounced thymus hypertrophy was observed in the exposed animals of the third and fourth generations. On the contrary, this was not encountered in the second-generation rats. Offspring's numbers were especially decreased fourth-generation according to second-generation (Figure 1). In addition, the average body masses of both female and male offspring in all generations were traced for five weeks. The average body masses of offspring were decreased in the fourth-generation (Figure 4).

Histopathological finding

In the slices stained with hematoxylin and eosin, the thymus is surrounded by a thin capsule consisting of connective tissue. Trabeculae extended from the capsule to the parenchyma of the organ. The thymus formed lobules that had apparent cortex and medulla. The organ cortex was more basophilic due to developing T cells, while the medulla was stained lighter. In each lobe, the thymus cortex was formed multiple lymphocytes and reticuloepithelial cells. The medulla of the organ consisted of numerous mature lymphocytes, reticuloepithelial cells, and Hassall corpuscles. In all groups, both second and third generation, cortex, medulla and the residing cells were noticeable (Figures 5 and 6). Although morphological differences did not appear among the groups, vascular structures were remarkable

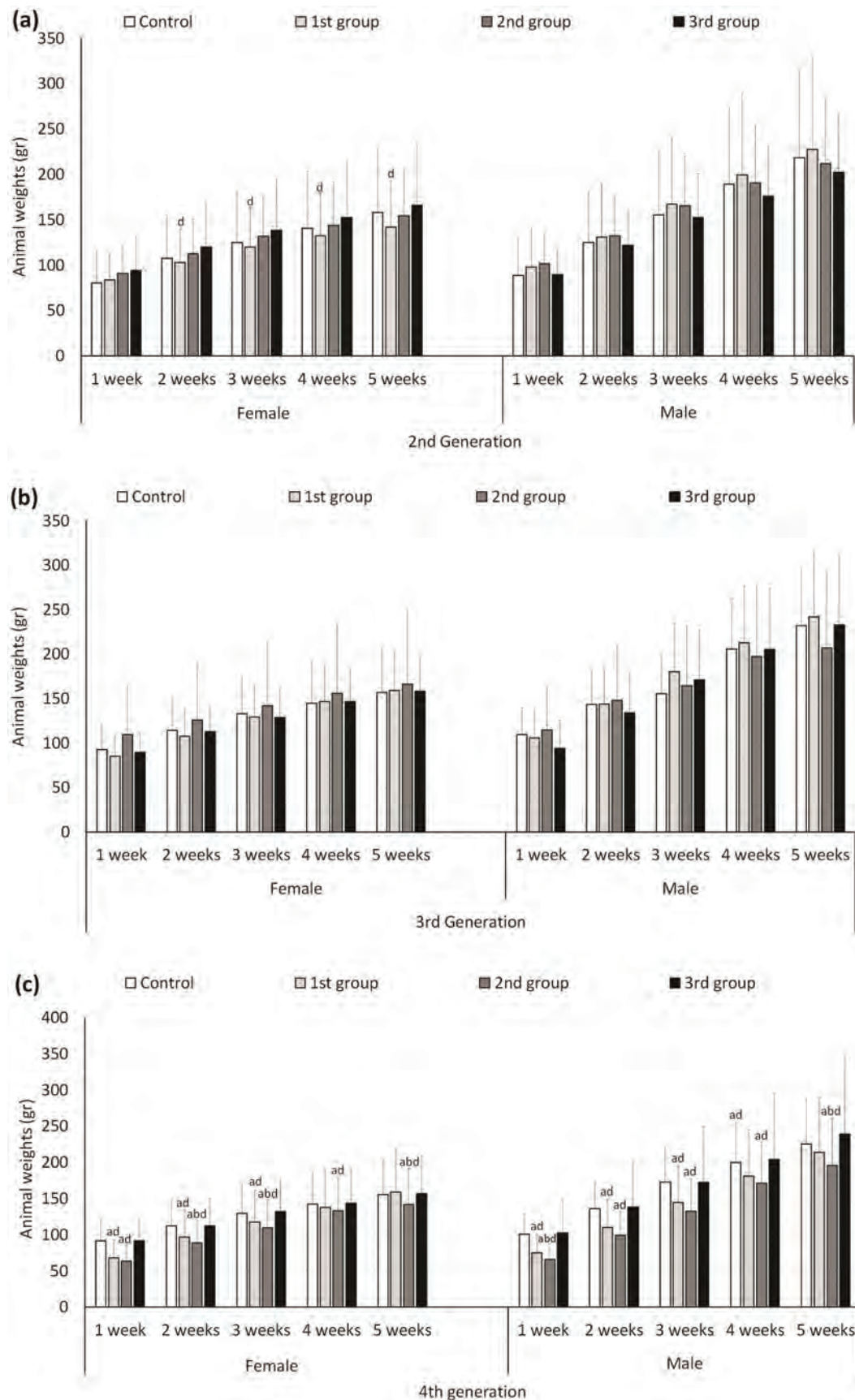


Figure 4. The average body masses of both female and male offspring in (a) second, (b) third and (c) fourth generations were traced for five weeks. The average body masses of offspring were decreased in the fourth-generation. Note that there was a significant difference between the groups ($p = .006$) in 2nd and 4th generation. a indicates significant difference compared to control group. b indicates significant difference compared to 1st group. d indicates significant difference compared to 3rd group.

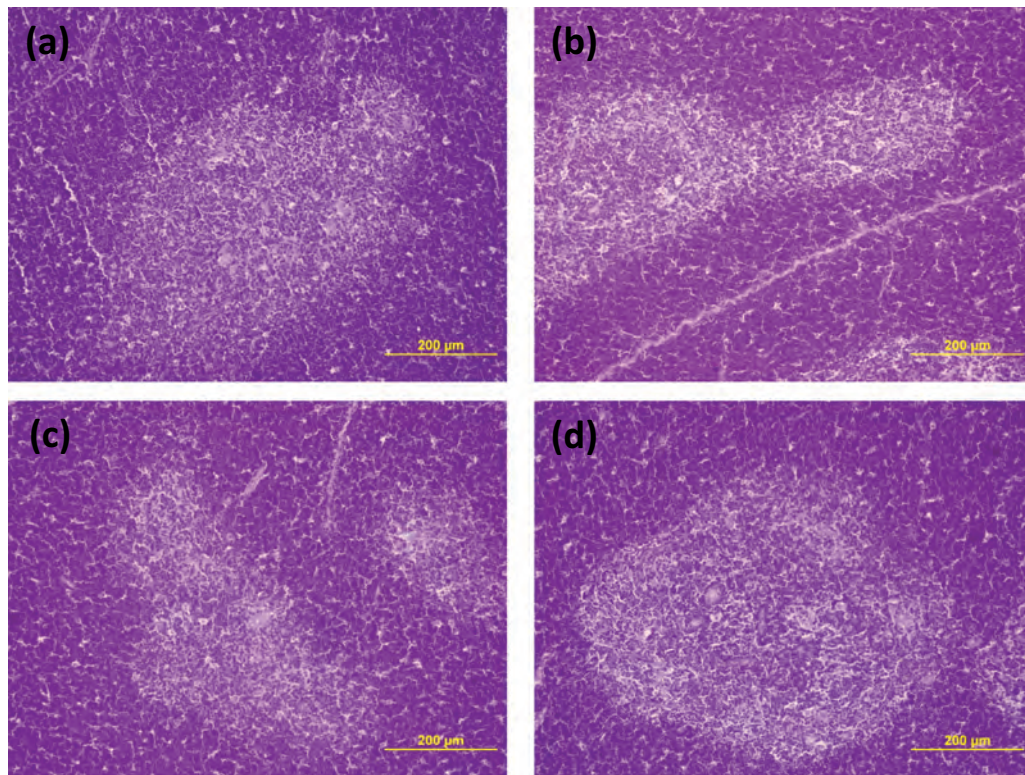


Figure 5. Histopathological changes in the thymus belonging to second generation rats exposed to EMF. In all groups of second generation; cortex, medulla and the residing cells were noticeable. The medulla of the organ consisted of numerous mature lymphocytes, reticuloepithelial cells and Hassall corpuscles. (a) Control group, (b) first group, (c) second group, (d) third group (H&E staining x400, Olympus, BX51, Tokyo, Japan).

when the second group of the fourth-generation was compared with the other experimental groups (Figure 7).

Discussion

The thymus is a fundamental lymphoid organ responsible for the maturation and selection of T cells. Thymus size and function are believed to decline with age (Gui et al. 2012). The thymus reaches its peak size during puberty, after which it gradually atrophies. If the thymus hypertrophy is not dependent on the patient's age, further investigation should be done (Khan and Anjum 2020). Thymic epithelial tumors manifest lobulated, contour, mediastinal fat invasion, hypertrophy, and vascular structure (Nishino et al. 2006).

This study investigated the effects of pre and postnatal 2450 MHz CWRFR on rats' thymus spanning four generations. Our study found morphological changes in the thymus tissue. Our most critical histopathological findings were visibly pronounced thymus hypertrophy in the exposed animals of the third and fourth generation and

the striking vascular structures in the second group of the fourth generation compared to the other experimental groups.

The results of this study were also consistent with earlier findings. Lee et al. investigated the effects of RF-EMF on AKR/J mice used for animal models of lymphoma. The mice were exposed to RF-EMF for 45 min/day, 5 days/week, over a total of 42 weeks. The thymus showed significant enlargement in most mice, and the thymoma was abnormal tissue (Lee et al. 2011). Misa-Agustino et al. exposed different levels of microwave radiation on rats. They reported histopathological changes induced 2450 MHz EMF in rat thymus. The researchers found extravascular erythrocytes in thymic tissue of animals exposed to EMF as in our study. Furthermore, they also reported an increased number of blood vessels in the hemorrhagic reticulo-epithelial cells and medulla (Misa-Agustino et al. 2015). It is evident from our results and the findings of Misa-Agustino et al. that EMF can cause changes in the morphology of thymic tissue.

Hanci et al. reported that light microscopy and transmission electron microscopy demonstrated pathological changes in splenic and thymic tissues of 900 MHz EMF

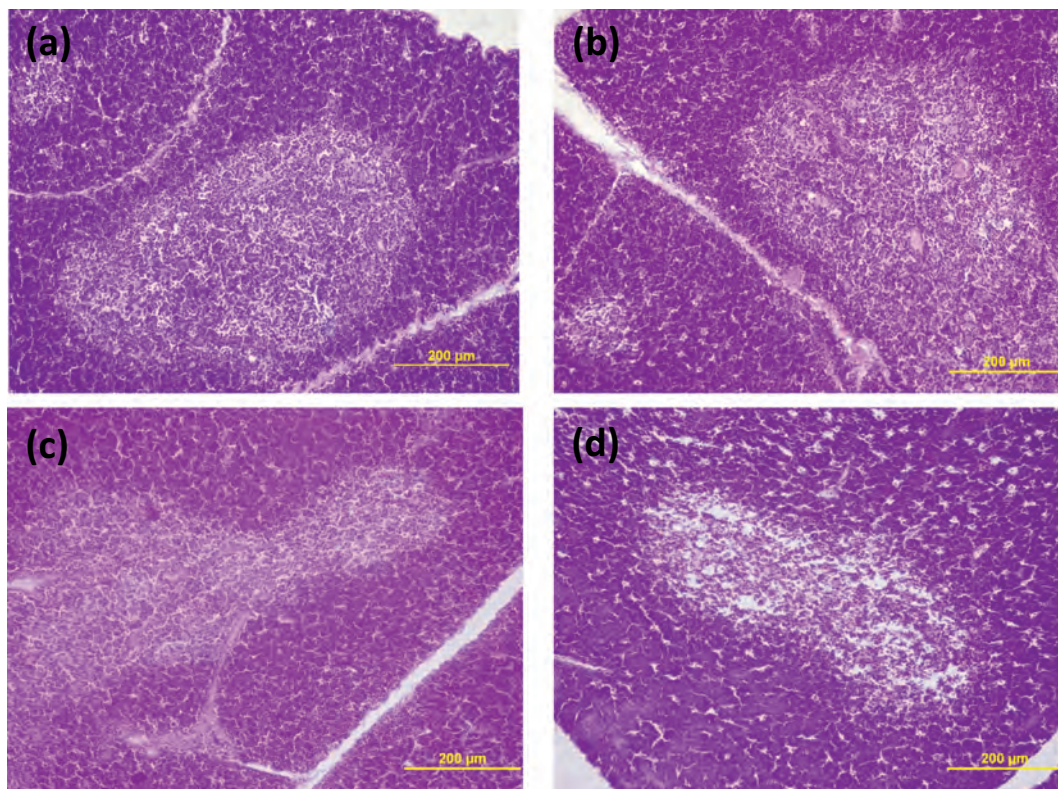


Figure 6. Histopathological changes in the thymus belonging to third generation rats exposed to EMF. In all groups of third generation; cortex, medulla and the residing cells were noticeable. The medulla of the organ consisted of numerous mature lymphocytes, reticuloepithelial cells and Hassall corpuscles. (a) Control group, (b) first group, (c) second group, (c) third group (H&E staining x400, Olympus, BX51, Tokyo, Japan).

exposed on rat offspring during the prenatal period. It was found that exposure during the prenatal period altered the antioxidant defense system and caused oxidative stress by increasing lipid oxidation. These researchers also reported mitochondrial degeneration, vacuolization, necrotic thymic lymphocytes, and epithelial cells in slices of EMF-exposed offspring (Hanci et al. 2015). Hashish et al. reported the effects of continuous whole-body exposure to both fields for 30 days on several blood and liver parameters in mice. They applied two magnetic fields: an extremely low-frequency electromagnetic field (ELF-EMF) and a static magnetic field (SMF). A significant reduction in the number of platelets, peripheral lymphocytes, monocytes, splenic total, T, and B lymphocyte levels was evaluated in the groups exposed to SMF and ELF-EMF (Hashisha et al. 2008). Kulaber et al. investigated changes in the thymic tissue of male rats exposed to continuous 900 MHz EMF for 1 h/day on postnatal days 22–59. They reported that a continuous 900 MHz EMF can change histopathology and increase the tissue MDA in male rat thymic tissue (Kulaber et al. 2017). Hekmat et al. applied 940 MHz RF-EMF on calf thymus DNA and stated that 940 MHz RF-EMF could alter the structure of DNA (Hekmat et al.

2013). Other researchers also reported the effects of ELF-EMF on the thymus and spleen tissues. Zhang et al. indicated that exposure to ELF-EMF in male mice decreased incidents of spleen and thymus tissues. Also, the exposure of ELF-EMF significantly decreased the NK-cell activity and proliferation of T and B lymphocytes (Zhang et al. 2016).

Contrary to our findings, some studies did not observe any change (Ohtani et al. 2015; Sommer et al. 2007, 2004). Sommer et al. (2004) exposed female AKR/J mice to 900 MHz EMF for 24 h/day, 7 days/week. Lymphoma incidence did not differ between the sham-exposed and exposed mice. Sommer et al. showed whether chronic exposure to the UMTS (Universal Mobile Telecommunication System) EMFs influenced the development of lymphoma in a lymphoma AKR/J mouse model. Exposure for 24 h/day, 7 days/week with an average whole-body SAR of 0.4 W/kg did not affect the lymphoma incidence and the malignancy of the lymphomas, which were genetically predisposed to this disease. Ohtani et al. reported the effects of continuous exposure to 2.14-GHz RF-EMF on T cell responses and its effects on the developmental immune systems. Their data indicated that long-term RF-EMF exposure had no

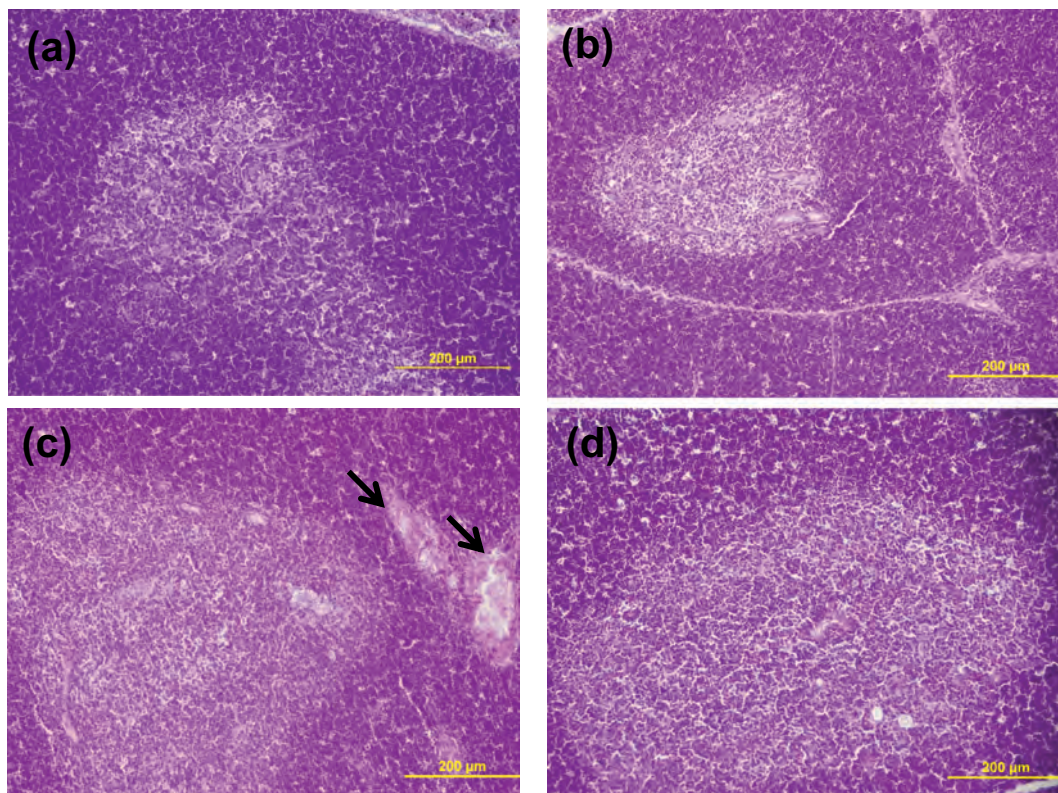


Figure 7. Histopathological changes in the thymus belonging to fourth generation rats exposed to EMF. The thymus cortex was formed multiple lymphocytes and reticuloepithelial cells. In first and third groups of fourth generation; cortex, medulla and the residing cells were noticeable. (a) Control group, (b) first group (c) in the second group, vascular structures were remarkable compared with other experimental groups, (d) third group (arrow; blood vessel, H&E staining x400, Olympus, BX51, Tokyo, Japan).

adverse impactation T cell activation, Th1/Th2 balance, and immune-like T cell populations in developing rats. However, important transcriptional effects were shown (Ohtani et al. 2015).

Conclusion

Our findings reveal that the effect of pre- and postnatal 2450 MHz CWRFR decreases the average body masses of rats. Pre- and postnatal 2450 MHz CW RFR exposure may potentially affect the thymus of future generations. Throughout three generations, increased vascularization and hypertrophy of the thymus can cause thymic epithelial tumors.

Disclosure statement

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Author contributions

F.C.T. and S.D. are responsible for the study design and writing of the article. B.Y. and A.H.Y. performed the microscopic analyses of slices. B.T. was responsible for the statistical analysis of the data. K.Y. was measured the SAR value.

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Effects of 3.5 GHz radiofrequency radiation on ghrelin, nesfatin-1, and irisin level in diabetic and healthy brains

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ABSTRACT

Diabetes, mobile phone use, and obesity have increased simultaneously in recent years. The radiofrequency radiation (RFR) emitted from mobile phones is largely absorbed in the heads of users. With 5 G, which has started to be used in some countries without the necessary precautions being taken, the amount of RFR to which living things are exposed will increase. In this study, the changes in energy homeostasis and redox balance caused by 5 G (3.5 GHz, GSM-modulated) were explored. The effects of RFR on the brains of diabetic and healthy rats were investigated and histopathological analysis was performed. Twenty-eight Wistar albino rats weighing 200–250 g were divided into 4 groups as sham, RFR, diabetes, and RFR+diabetes groups (n = 7). The rats in each group were kept in a plexiglass carousel for 2 h a day for 30 days. While the rats in the experimental groups were exposed to RFR for 2 h a day, the rats in the sham group were kept under the same experimental conditions but with the radiofrequency generator turned off. At the end of the experiment, brain tissues were collected from euthanized rats. Total antioxidant (TAS), total oxidant (TOS), hydrogen peroxide (H₂O₂), ghrelin, nesfatin-1, and irisin levels were determined. In addition, histopathological analyses of the brain tissues were performed. The specific absorption rate in the gray matter of the brain was calculated as 323 mW/kg and 195 mW/kg for 1 g and 10 g averaging, respectively. After RFR exposure among diabetic and healthy rats, decreased TAS levels and increased TOS and H₂O₂ levels were observed in brain tissues. RFR caused increases in ghrelin and irisin and a decrease in nesfatin-1 in the brain. It was also observed that RFR increased the number of degenerated neurons in the hippocampus. Our results indicate that 3.5 GHz RFR causes changes in the energy metabolism and appetite of both healthy and diabetic rats. Thus, 5 G may not be innocent in terms of its biological effects, especially in the presence of diabetes.

1. Introduction

Rapid developments in wireless communication technologies, especially mobile phones, and the intensive use of these instruments by people of all ages have led scientists to focus their attention on the human health effects of the radiofrequency radiation (RFR) emitted by these technologies. While 2 G, 3 G, 4 G, and 4.5 G technologies use radiofrequency waves of 900 MHz, 1800 MHz, and 2100 MHz, the most preferred radiofrequency band for 5 G technology, which has begun to be used in many countries in recent years, is 3.5 GHz (Dasgupta et al.,

2020; Wang et al., 2022). With the innovations offered by 5 G technology and the application of the internet of things, the RFR exposure time and intensity of living things are increasing. The RFR emitted from mobile phones is not sufficient for the ionization of molecules and the activation of orbital electrons. It is now known that the underlying mechanism of DNA and other cellular elements affected by RFR is oxidative stress (Dasdag and Akdag, 2016; Yakymenko et al., 2016). It has been suggested that RFR (2.45 GHz, with 217 Hz pulses, during 60 min/day for 30 days, whole-body SAR 0.1 W/kg) can change cation transitions by affecting voltage-dependent channels in DRG neurons and

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brains of Wistar Albino rats (Naziroğlu et al., 2012). The use of 5 G with other currently used generations of technology standards with different frequencies will lead to accumulative effects (Tan et al., 2017). Cumulative biological effects are strongly related to the intensity and duration of radiofrequency exposure. The current exposure limits are based on adverse health outcomes due to temperature rise (tissue warming and inducible tissue stimulation from short-term or acute exposures) (Bel-pomme et al., 2018). There are no precautions for chronic and/or cumulative health risks associated with radiofrequency energy at substandard levels (Dolan and Rowley, 2009). Changes mediated by reactive oxygen species (ROS) are the most convincing mechanisms put forward to explain the effects of RFR on living things (Dasdag and Akdag, 2016; Yakymenko et al., 2016). Because the brain, a vital organ, is particularly close to the source of RFR exposure, studies on the effects of RFR have mostly focused on the brain and the brain was shown to be affected by RFR (Kim et al., 2021; Tripathi et al., 2021). In addition, brain tissues are more vulnerable to oxidative damage due to their low levels of antioxidant enzyme activity and high oxygen consumption (Motawi et al., 2014). There are a number of studies reporting that exposure to RFR at various specific absorption rate (SAR) levels causes oxidative stress in the brain (Avci et al., 2012; Chauhan et al., 2017; Chen et al., 2011; Kesari et al., 2011; Shehu et al., 2016; Singh et al., 2020). Other studies have revealed that the basis of age-related neurodegenerative diseases is imbalance in redox mechanisms (Sahin et al., 2016).

Diabetes mellitus (DM) is a disease that is spreading rapidly around the world due to the living conditions of people today and it carries with it high risks of mortality and morbidity. Studies reported that ROS and lipid peroxidation are significantly increased in rats with experimentally induced diabetes and in human patients with diabetes, and oxidative stress has a role in the etiology and progression of diabetes (Wang et al., 2018). DM is associated with increased ROS production, inadequate antioxidant defense mechanisms, and, consequently, increased oxidative stress (Bathina and Das, 2021).

Studies on mediators that have effects on nutrition in humans and animals have led researchers to focus on nesfatin and ghrelin. These are hormones that influence food intake, while irisin is one of the hormones that are effective in regulating energy metabolism (Price et al., 2007). It is accepted that appetite is controlled by the brain and eating is regulated by complex mechanisms in the central nervous system and particularly in the hypothalamus (Druce and Bloom, 2003). The level of ghrelin is increased by fasting and it decreases within 60–120 min following food intake (Tschöp et al., 2001). In rats, it was shown that fasting increased ghrelin release, while carbohydrate intake decreased ghrelin release (Cummings et al., 2001). It is thought that adipose tissue and appetite-enhancing effects of ghrelin are regulated by special neurons in the central nervous system (Tschöp et al., 2001).

It is known that nesfatin plays roles in the brain not only in the regulation of food intake but also in the regulation of some brain functions, autonomic regulation, stress, mood, and REM sleep (Jego et al., 2012).

Irisin is a hormone attracting the attention of researchers since its recent discovery; it is thought to have potential in the treatment of many metabolic diseases in the future, especially obesity and diabetes, and it is a highly effective peptide in neurodegeneration (Moreno-Navarrete et al., 2013).

In a recent human study, 15 normal-weight young individuals (23.47 ± 0.68 years) were exposed to sham radiation and two different types of mobile phones emitting 900 MHz RFR (0.97 W/kg and 1.33 W/kg) under fasting conditions for 25 min. Afterwards, spontaneous food intake was determined by an ad libitum standard buffet test and cerebral energy homeostasis was monitored by phosphorus-magnetic resonance spectroscopy measurements. It was reported that exposure to both types of mobile phones strikingly increased the overall caloric intake by 22–27 % compared to the sham condition (Wardzinski et al., 2022). Moreover, measurement results of cerebral energy contents, such as the ratios of

adenosine triphosphate and phosphocreatine to inorganic phosphate, showed increases with cell phone radiation exposure (Wardzinski et al., 2022). These researchers argued that RFR is a potential contributor to the overeating that underlies the obesity epidemic (Wardzinski et al., 2022). Furthermore, pulsed and amplitude modulated microwaves were shown to affect brain energy homeostasis in rats (Sanders et al., 1985). It was also demonstrated that both the brain and energy homeostasis are affected by RFR (Tripathi et al., 2021). Based on these previous findings, we hypothesized that 3.5 GHz RFR exposure affects irisin, ghrelin, and nesfatin-1 levels via oxidant and antioxidant redox systems in the brain.

The present study was designed to determine the effects of exposure to 3.5 GHz RFR (GSM, 217 Hz modulation frequency, pulse width 577 µs) emitted from 5 G technologies on total antioxidant (TAS), total oxidant (TOS), hydrogen peroxide (H₂O₂), nesfatin, ghrelin, and irisin levels in the brains of healthy and diabetic rats and possible histological changes.

2. Materials and methods

2.1. Animal care

This experimental study was carried out in the Experimental Research and Application Center of Van Yuzuncu Yil University. All stages of the study were designed and performed according to the guidelines of the Animal Experiments Local Ethics Committee of Van Yuzuncu Yil University (Protocol No: 2021/05–10).

A total of 28 healthy male Wistar albino rats (8–10 weeks old and weighing 200–250 g) were obtained from the Experimental Animal Research Center of Van Yuzuncu Yil University. The rats were separated and placed in four stainless steel cages with seven rats per cage and were maintained with a 12-hour dark/light cycle. Rats were allowed free access to pellet feed (Purina, Istanbul, Turkey) containing 21 % crude protein and daily drinking water ad libitum in optimal environmental conditions (22 ± 2 °C and 60 ± 5 % humidity).

2.2. Animal grouping and protocol

This study included four different groups of rats (n = 7 for each group). In Group 1 (healthy sham group), healthy rats (n = 7) were kept in a plexiglass carousel for 2 h a day for 30 days while the radio-frequency generator was turned off. In Group 2 (healthy RFR group), healthy rats (n = 7) were irradiated with radiofrequency signals (3.5 GHz, GSM-modulated) in the plexiglass carousel for 2 h a day for 30 days. In Group 3 (diabetes sham group), rats with diabetes were kept in the plexiglass carousel for 2 h a day for 30 days while the generator was turned off. In Group 4 (RFR+diabetes group), rats with diabetes were irradiated with RFR (3.5 GHz, GSM-modulated) for 2 h a day for 30 days (n = 7).

2.3. Induction of experimental diabetes

Streptozotocin (STZ) is an antibiotic that produces pancreatic islet β-cell destruction and is widely used experimentally to produce models of type 1 DM (Furman, 2015). To induce experimental diabetes, rats in Groups 3 and 4 were fasted overnight and then 45 mg/kg of STZ was administered intraperitoneally in a single dose. Blood samples were taken from rats 72 h after STZ application to confirm the presence of DM. Blood glucose levels were measured by a glucometer (IME-DC) and blood glucose levels above 250 mg/dL were considered as diabetic (Öntürk and Özbek, 2007).

2.4. Exposure and field measurements

A GSM signal generator (3500 PM10 model, Everest Comp., Adapazari, Turkey) that produces a 3500 MHz band (GSM-modulated) radiofrequency waveform identical to that of mobile phones was used in

this study to expose the rats to RFR. The emitted power (omnidirectional on the plane perpendicular to the antenna axis) of the generator was fixed during exposure. The antenna of the generator was equivalent to that of a typical mobile phone. Rats were confined in a plexiglass carousel and exposed to 3500 MHz RFR emitted from the generator. The carousel was surrounded with electromagnetic absorber material backed by metal to isolate outdoor electromagnetic fields from the test setup during the study duration of 1 month. Power density and the electrical field were measured with a TES 593 field probe (Shenzhen, Guangdong, China). The measured electric field and power density values were 28.1 V/m, 1.6 W/m². The rats in the exposure groups were exposed to RFR for 2 h per day (5 days a week) for 1 month. For the sham groups, rats were placed in the carousel and the same procedure was applied except that the generator was turned off, i.e., no radiofrequency signal was present. The antenna of the generator was placed at the center of the plexiglass carousel to provide ideal exposure conditions. The distance of the antenna from the heads of the rats was 1 cm (Alkis et al., 2019). All rats were kept under identical conditions for 1 month with free access to food and water. The experimental setup is illustrated in Fig. 1.

2.5. Specific absorption rate (SAR) analysis

The simulation setup was created to match the experimental setup as much as possible. The applicator antenna, the Faraday cage, and the rats

were all modeled in a 3D electromagnetic field solver using CST (CST AG, Darmstadt, Germany) (Alkis et al., 2019). The simulation model is depicted in Fig. 1. An accurate rat model based on computerized tomography scans is readily available for biological CST applications. This rat voxel (volumetric pixel) model is very detailed and includes all tissues within the rat. While peak power of 2 W is common for GSM burst signals, average power for SAR evaluations is taken as 250 mW. Electromagnetic and thermal modeling within CST is based on the finite-integration technique, which is a combined method of integral and differential solvers acting in space and time, respectively. Simulated electric field values were also compared to measured field values with the TES 593 field probe to validate the simulation setup.

2.6. Sample collection and preparation of brain tissue homogenates

At the end of the experiment, under ketamine (Ketasol, Richter Pharma AG, Vienna, Austria) and bacillazine (Rompin, Istanbul, Turkey) anesthesia, the rats were killed by cervical dislocation and the brains were removed immediately. Part of the brain was used for the preparation of a homogenate. The brain tissues were carefully washed and weights were recorded. The 10 % homogenate was prepared in phosphate-buffered saline (PBS) and centrifuged at 10,000g for 10 min. The supernatant was collected for further biochemical assays. Supernatants from brain homogenates were not frozen and analyzed immediately after the study.

2.7. Nesfatin-1 analyses

The kit used in this study is a sandwich enzyme immunoassay for the in vitro quantitative measurement of nesfatin-1 (SunRed Biological Technology Co., Ltd., Shanghai, China, Catalog No: 201-11-1426). Each well of the supplied microtiter plate is precoated with a target-specific capture antibody. Standards and samples are added to the wells and the target antigen binds to the NES-1 antibody labeled with biotin. Afterwards, a streptavidin-HRP conjugate, chromogen A and B substrate, and stop solution are added to terminate color development reactions and then the optical density of the wells is measured at a wavelength of 450 ± 2 nm using automated optical densitometry (intra-assay CV: <8 %, inter-assay CV: <11 %, sensitivity: 28.951 ng/L, assay range: 30–9000 ng/L). Each sample was run in duplicate and the mean value was used for analysis. This assay has high sensitivity and excellent specificity for detection of rat nesfatin-1. No significant cross-reactivity or interference between rat nesfatin-1 and analogues was observed (Algul et al., 2021).

2.8. Ghrelin analysis

The kit used in this study is a sandwich enzyme immunoassay for the in vitro quantitative measurement of ghrelin (SunRed Biological Technology Co., Ltd., Shanghai, China, Catalog No: 201-11-1650). Each well of the supplied microtiter plate is precoated with a target-specific capture antibody. Standards and samples are added to the wells and the target antigen binds to the ghrelin antibody labeled with biotin. Afterwards, a streptavidin-HRP conjugate, chromogen A and B substrate, and stop solution are added to terminate color development reactions and then the optical density of the wells is measured at a wavelength of 450 ± 2 nm using automated optical densitometry (sensitivity: 38.572 pg/mL, assay range: 40–10000 pg/mL). Each sample was run in duplicate and the mean value was used for analysis. This assay has high sensitivity and excellent specificity for detection of rat ghrelin. No significant cross-reactivity or interference between rat ghrelin and analogues was observed (Kızılırmak et al., 2020).

2.9. Irisin analysis

The kit used in this study is a sandwich enzyme immunoassay for the

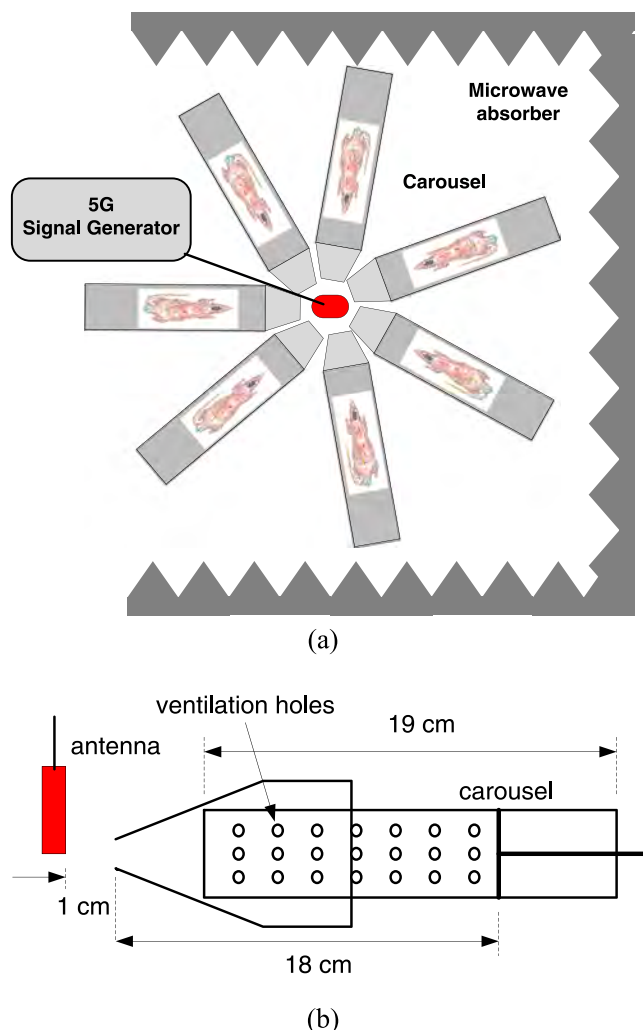


Fig. 1. Experimental setup: a) exposure setup, b) plexiglass carousel and antenna.

in vitro quantitative measurement of irisin (SunRed Biological Technology Co., Ltd., Shanghai, China, Catalog No: 201-11-1713). Each well of the supplied microtiter plate is precoated with a target-specific capture antibody. Standards and samples are added to the wells and the target antigen binds to the irisin antibody labeled with biotin. Afterwards, a streptavidin-HRP conjugate, chromogen A and B substrate, and stop solution are added to terminate color development reactions and then the optical density of the well is measured at a wavelength of 450 ± 2 nm using automated optical densitometry (sensitivity: 0.247 ng/mL, assay range: 0.3–90 ng/mL). Each sample was run in duplicate and the mean value was used for analysis. This assay has high sensitivity and excellent specificity for detection of rat irisin. No significant cross-reactivity or interference between rat irisin and analogues was observed (Akdog and Odabas, 2020).

2.10. Total antioxidant status (TAS) analysis

The kit used in this study is a sandwich enzyme immunoassay for the in vitro quantitative measurement of TAS (SunRed Biological Technology Co., Ltd., Shanghai, China, Catalog No: 201-11-2672). Each well of the supplied microtiter plate is precoated with a target-specific capture antibody. Standards and samples are added to the wells and the target antigen binds to the TAS antibody labeled with biotin. Afterwards, a streptavidin-HRP conjugate, chromogen A and B substrate, and stop solution are added to terminate color development reactions and then the optical density of the wells is measured at a wavelength of 450 ± 2 nm using automated optical densitometry (intra-assay CV: <9 %, inter-assay CV: <11 %, sensitivity: 0.175 U/mL, assay range: 0.2–60 U/mL). Each sample was run in duplicate and the mean value was used for analysis. The sensitivity and specificity of assay for detection of rat TAS is in acceptable ranges.

2.11. Total oxidant status (TOS) analysis

The kit used in this study is a sandwich enzyme immunoassay for the in vitro quantitative measurement of TOS (SunRed Biological Technology Co., Ltd., Shanghai, China, Catalog No: 201-11-1669). Each well of the supplied microtiter plate is precoated with a target-specific capture antibody. Standards and samples are added to the wells and the target antigen binds to the TOS antibody labeled with biotin. Afterwards, a streptavidin-HRP conjugate, chromogen A and B substrate, and stop solution are added to terminate color development reactions and then the optical density of the wells is measured at a wavelength of 450 ± 2 nm using automated optical densitometry (intra-assay CV: <8 %, inter-assay CV: <10 %, sensitivity: 0.073 nmol/mL, assay range: 0.1–20 nmol/mL). Each sample was run in duplicate and the mean value was used for analysis. The sensitivity and specificity of assay for detection of rat TOS is in acceptable ranges.

2.12. H_2O_2 analysis

The kit used in this study is a sandwich enzyme immunoassay for the in vitro quantitative measurement of H_2O_2 (SunRed Biological Technology Co., Ltd., Shanghai, China, Catalog No: 201-11-3366). Each well of the supplied microtiter plate is precoated with a target-specific capture antibody. Standards and samples are added to the wells and the target antigen binds to the H_2O_2 antibody labeled with biotin. Afterwards, a streptavidin-HRP conjugate, chromogen A and B substrate, and stop solution are added to terminate color development reactions and then the optical density of the wells is measured at a wavelength of 450 ± 2 nm using automated optical densitometry (sensitivity: 9.005 ng/mL, assay range: 10–3000 ng/mL). Each sample was run in duplicate and the mean value was used for analysis. This assay has high sensitivity and excellent specificity for detection of rat H_2O_2 . No significant cross-reactivity or interference between rat H_2O_2 and analogues was observed (Kamiş and Karabağ, 2019).

2.13. Immunohistochemical staining

After another part of the brain was fixed in 10 % buffered formaldehyde, the samples were embedded in paraffin following routine histological processing steps. Sections of 4 μ m in thickness were taken from paraffin blocks with a microtome and placed on lysine slides. Sections were deparaffinized and rehydrated, and then incubated in 3 % H_2O_2 for 10 min to prevent endogenous peroxidase enzyme activity. To prevent antigen masking in the nucleus, they were heated in antigen retrieval solution (citrate buffer, pH 6.1) in a microwave oven twice. Then, to prevent nonspecific binding, the protein block was dropped and incubated for 10 min. Anti-synaptophysin antibody (Santa Cruz Biotechnology, sc-365447, dilution: 1/100) was used as the primary antibody. After adding the antibody, these samples were incubated in a humidity chamber at 4 °C for one night. After rinsing with PBS, biotinylated goat anti-polyvalent and streptavidin-peroxidase conjugate were dropped and incubated for 10 min, respectively. After the application of diaminobenzidine (DAB), used as a chromogen, samples were stained with Mayer's hematoxylin and examined under a light microscope (Olympus BX53, Olympus, Japan) (Altundağ et al., 2021). Immunohistochemical findings were assessed as negative (-), slightly positive (+), moderately positive (++), and intensely positive (+++) according to the immunostaining characteristics of the cells.

2.14. Cresyl violet staining method

To evaluate neuronal damage in the hippocampus of rats exposed to 5 G and induced diabetes, the 0.1 % cresyl violet staining method, which specifically stains Nissl bodies, was applied. Brain sections taken at 5 μ m in thickness were deparaffinized, dehydrated, and stained with cresyl violet for 30 min. Neurons stained with cresyl violet were evaluated and photographed by a blinded histologist under a light microscope (Olympus BX53, Olympus, Japan) according to low and high staining intensity (Jin and Bo, 2021; Justin et al., 2018; Tóthová et al., 2018).

2.15. Statistical analysis

The SPSS statistical software program was used in the analyses. Whether the data of each group originated from the same distribution was tested by rank-based Kruskal-Wallis tests or one-way ANOVA. For comparisons of groups, the Mann-Whitney test was used to determine if there was a statistically significant difference between them. The results were presented as medians and $p < 0.05$ was taken to be statistically significant. In tables, the groups were labeled with letters to indicate differences such that if a group has a different letter than others a statistically significant difference exists.

3. Results

3.1. Results of body weight

While the average initial weight of the healthy rats was 233.5 g, it was increased to 302.5 g at the end of the RFR exposure ($p = 0.004$).

Table 1
Body weights of the rats.

Groups	Weight (g)	p
Healthy sham (start)	237.5 $\pm$ 21.9 ^a	0.128
Healthy sham (end)	261 $\pm$ 25.4 ^a	
Healthy RFR (start)	233.5 $\pm$ 11.2 ^a	
Healthy RFR (end)	302.5 $\pm$ 113.8 ^b	0.004
Diabetes sham (start)	281.5 $\pm$ 8.12 ^a	
Diabetes sham (end)	254 $\pm$ 26.1 ^b	
Diabetes RFR (start)	267.5 $\pm$ 18.1 ^a	0.055
Diabetes RFR (end)	249 $\pm$ 21.7 ^a	

^{a,b}: Different letters indicate significant differences between groups.

There was no significant change in the weights of the diabetic rats after 5 G exposure (Table 1).

3.2. SAR results

SAR calculations based on the IEEE/IEC 62704-1 method were used to evaluate the SAR distribution. Whole-body SAR was found to be 37 mW/kg. SAR of the gray matter of the brain was calculated as 323 mW/kg and 195 mW/kg for 1 g and 10 g averaging, respectively. The maximum SAR of the blood and blood vessels was 162 mW/kg and 178 mW/kg for 1 g and 10 g averaging, respectively. In fat tissues, SAR reached maximum values of 220 mW/kg and 184 mW/kg for 1 g and 10 g averaging, respectively. The rat voxel model, rat brain, blood vessels, and blood are shown in Fig. 2. SAR distributions for 1 g and 10 g averaging on different cut-planes are displayed in Fig. 3.

3.3. ELISA results

There was a significant difference between the groups for levels of ghrelin in the brain ($p = 0.001$). Ghrelin levels in the brain were increased in Group 2 compared to Group 1 and in Group 4 compared to Group 3 (Table 2).

There was a significant difference between the groups in nesfatin-1 levels in the brain ($p = 0.018$). Nesfatin-1 levels in the brain were decreased in Group 2 compared to Group 1 and in Group 4 compared to Group 3 (Table 2).

There was a significant difference between the groups in the level of irisin in the brain ($p = 0.001$). Irisin levels in the brain were decreased in Group 2 compared to Group 1 and in Group 4 compared to Groups 1 and 2 (Table 2).

There was a significant difference between the groups in the TAS of the brain ($p = 0.002$). TAS values of the brain were decreased in Group 2 compared to Group 1 and in Group 4 compared to Group 3 (Table 2).

There was a significant difference between the groups in the TOS of the brain ($p < 0.001$). TOS values of the brain were increased in Group 2 compared to Group 1 and in Group 4 compared to Group 3 (Table 2).

There was a significant difference between the groups in the level of H_2O_2 in the brain ($p < 0.001$). H_2O_2 levels in the brain were increased in Group 2 compared to Group 1 and in Group 4 compared to Group 3 (Table 2).

There was no difference between the groups in terms of anti-synaptophysin expression (Fig. 4).

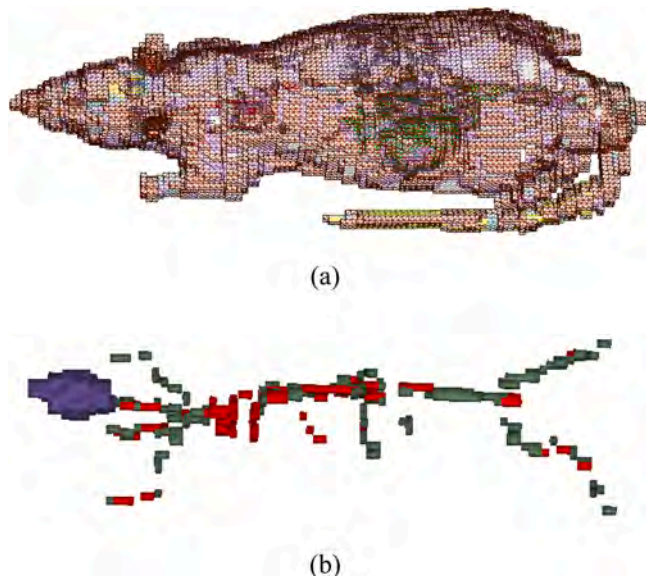


Fig. 2. a) Rat voxel model; b) gray matter, blood and blood vessels.

The intensity of cresyl violet staining, which we performed to evaluate the intensity of neuronal degeneration, was lower in Group 1. High cresyl violet staining intensity was found in Groups 2, 3, and 4, and it was particularly intense in Group 2 (Fig. 5).

4. Discussion

There is growing concern about increasing electromagnetic pollution due to RFR emitted from wireless devices. Exposure to RFR varies from individual to individual according to the length of time and frequency of use of such devices. The 3.5 GHz band is one of the frequency bands used in 5 G technology (Dasgupta et al., 2020; Wang et al., 2022) and it was consequently selected for the present study. Older wireless telephones use frequencies in the 900, 1800, and 2100 MHz ranges. The mechanisms induced by RFR remain unknown, but commonly accepted theories include changes in membrane permeability and ROS production (Naziroğlu et al., 2012).

The results of the present study showed that 3.5 GHz RFR caused a significant increase in TOS and H_2O_2 and a decrease in TAS in healthy rats, and these changes were more intense in the brains of diabetic rats. Our findings are in agreement with the results of previous studies that demonstrated RFR-induced oxidative stress in the brain (Chen et al., 2011; Ertilav et al., 2018) due to its polyunsaturated fatty acid-rich composition and high metabolic rate (Singh et al., 2020). Supporting these results, several in vivo studies found enhanced ROS formation in the brain after GSM RFR (1.6–6 W/kg) exposure with increased oxidative stress (Megha et al., 2015; Stefi et al., 2019; Zielinski et al., 2020). However, the level and extent of changes in oxidative stress parameters are specific to the dose and duration of exposure and the experimental setup. Megha et al. reported an increase in oxidative stress in the brains of male Fischer rats exposed to GSM modulated RFRs (900, 1800, 2100 MHz, for 60 days, SAR: 0.59, 0.58 and 0.66 mW/kg respectively) while shifting toward higher frequencies and SAR values (Megha et al., 2015). Taken together, the results of previous studies on this subject indicate that non-thermal RFR can be absorbed by particular charges, molecules, and cellular constructions; thus, they can stimulate important modulatory effects in living cells (Yakymenko et al., 2016). Kesari et al. reported that UMTS modulated RFR (2115 MHz, 2 h/day for 60 days SAR: 0.26 W/kg) originating from 3 G technology caused DNA breaks in the brain of the Wistar rats (Kesari et al., 2014). On the other hand, Dasdag et al., who suggested that RFR is an environmental stress factor, determined that long-term exposure to GSM modulated 900 MHz RFR (2 h per day for 10 months, SAR: 0.17–0.58 W/kg) caused increases in the total antioxidant capacity and catalase level in the brain of the Wistar Albino rats (Dasdag et al., 2009). Şahin et al. observed an increase in oxidative DNA damage in the brain of the Wistar albino rats after 10 days of exposure to 3 G modulated 2100 MHz RFR (6 h per day, 5 consecutive days per week for 2 weeks, SAR: 0.4 W/kg), while they found a decrease in oxidative DNA damage after 40 days of application (Şahin et al., 2016). The reason for the emergence of these different results was said to be the adaptation of the body to long-term RFR exposure and the activation of DNA repair mechanisms (Şahin et al., 2016). In another study, Dasdag et al. stated that long-term exposure to Wi-Fi 2.4 GHz RFR (24 h a day for 12 months, SAR: 141.4 μ W/kg and 7127 μ W/kg) caused changes in miRNAs in the brain of the Wistar Albino rats, which could be associated with the prognosis of some brain diseases (Dasdag et al., 2015). Moreover, Dasdag et al. found in another study that exposure to GSM modulated 900 MHz RFR (2 h per day, 7 days a week for 10 months, SAR: 0.17–0.58 W/kg) increased the levels of beta-amyloid protein, protein carbonyl, and malondialdehyde in brains of Wistar Albino rats (Dasdag et al., 2012). On the other hand, Arendash et al. reported that RFR (GSM modulated, 918 MHz, 1 h/day, for 5 and 7 months, SAR: 0.25 W/kg) provided cognitive benefits by reducing the amount of amyloid beta in the brains of transgenic and non-transgenic mice with Alzheimer disease (Arendash et al., 2010). Sharma et al. reported that GSM 1800 MHz RFR (SAR: 0.433 W/kg)

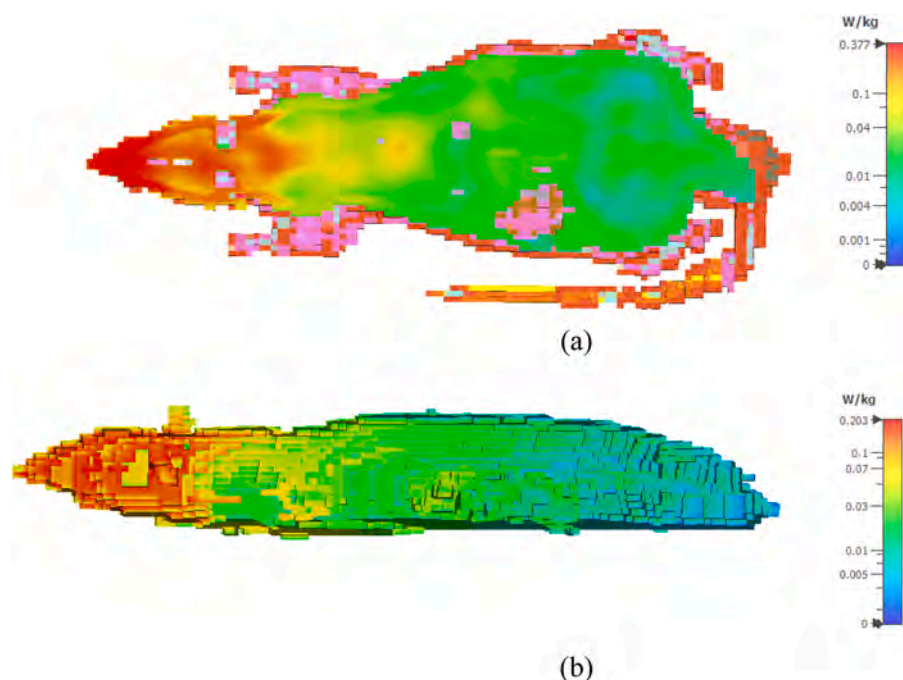


Fig. 3. SAR simulation results at 3500 MHz: a) 1 g average frontal plane, b) 10 g average sagittal plane.

Table 2
Ghrelin, nesfatin-1, irisin, TAS, TOS, and H_2O_2 in the brains of the rats.

	Healthy sham (Group 1)	Healthy + RFR (Group 2)	Diabetes sham (Group 3)	Diabetes + RFR (Group 4)	p
Ghrelin pg/mL	2967.7 $\pm 275.6^{ab}$	3421.8 $\pm 214.9^{ac}$	3327.6 $\pm 240.6^a$	3841.5 $\pm 253.9^d$	0.001
Nesfatin-1 ng/mL	1890.6 $\pm 221.9^a$	1687.7 $\pm 190.5^{ab}$	1684.3 $\pm 332.3^{ab}$	1405.4 $\pm 255.1^b$	0.018
Irisin ng/mL	31.2 $\pm 3.5^a$	25.5 $\pm 3.5^b$	14.8 $\pm 2.2^c$	12.3 $\pm 0.7^c$	0.001
TAS U/mL	10.5 $\pm 2.7^a$	9.6 $\pm 2.4^{ab}$	7.38 $\pm 0.98^{ac}$	5.00 $\pm 0.4^d$	0.002
TOS nmol/mL	0.23 $\pm 0.3^a$	2.5 $\pm 0.5^b$	3.719 $\pm 0.75^b$	6.4 $\pm 1.2^c$	<0.001
H_2O_2 ng/mL	378.2 $\pm 85.0^a$	477.1 $\pm 57.3^b$	726.259 $\pm 151.7^c$	1068.5 $\pm 152.4^d$	<0.001

a,b,c,d: Different letters indicate significant differences between groups (p < 0.05).

caused an increase in oxidative stress and inflammatory cytokine release in the brains of Wistar rats (90 days at 4 h per day 5 days per week in a month) (Sharma et al., 2020). In another study, Sharma et al. used RFR at frequencies of 900, 1800, and 2100 MHz and SAR levels of 0.434 W/kg, 0.433 W/kg, and 0.453 W/kg, respectively. When animal exposure was limited to 1 h per day, 5 days/week for 1 month, a significant decrease in antioxidant enzyme capacity, increased ROS levels, and degraded pyramidal and Purkinje neurons were observed in the brains of the Wistar rats (Sharma et al., 2021). Similarly, Chauhan et al. revealed that RFR (2.45 GHz radiation source at 50 Hz modulation frequency) exposure (2 h per day for 35 day, SAR: 0.14 W/kg) caused histological and oxidative changes, reductions in pyramidal cells, and neuronal degradation in the brains of Wistar rats (Chauhan et al., 2017). Lai and Singh stated that acute 2-h exposure to 2450-MHz RFR (pulsed, 2 μ s pulse width, 500 pulses s^{-1} and continuous wave, SAR: 1.2 W/kg) can cause single and double chain breaks in the brain of Sprague Dawley rats due to oxidative stress (Lai and Singh, 1996). One of the major sources of ROS in cells is the mitochondrial electron transport chain,

which can produce superoxide due to deteriorations in electron transport (Inoue et al., 2003). It was shown that formation of ROS by the mitochondrial pathway can be induced under GSM 1.8 GHz RFR exposure (SAR: from 0.4 W/kg to 27.5 W/kg) in human spermatozoa (De Iuliis et al., 2013). Taken together, the findings in the current literature on the biological effects of RFR convincingly demonstrate that exposure to low-intensity RFR causes the formation of substantial amounts of ROS in living cells and results in important oxidative stress (Yakymenko et al., 2016).

In our study, changes in the levels of nesfatin-1 and ghrelin, which are known as appetite hormones, were detected in the brains of rats exposed to RFR. More specifically, 5 G RFR caused a decrease in nesfatin levels and an increase in ghrelin levels in the brains of both diabetic and healthy rats. Recent studies have shown that nesfatin as an appetite suppressant (Shimizu and Mori, 2013) and ghrelin as an appetite stimulant (Morin et al., 2018) play active roles in adipose tissue accumulation and appetite mechanisms. That is, decreased nesfatin and increased ghrelin cause an increase in appetite. This indicates that exposure to 3.5 GHz RFR has an appetite-increasing effect on rats. Similarly, in a study in which they used RFR of a lower frequency, Tripathi et al. reported that RFR (2 h per day for 8 weeks, 1760 MHz, 0.3988 mW/cm², GSM, SAR: 0.181 W/kg) caused an increase in ghrelin levels in the brain of rats (Tripathi et al., 2021). It was stated that an increase in the amount of ghrelin causes an increase in appetite and thus the need for food (Tripathi et al., 2021). In addition, it was reported that in albino rats prolonged GSM 900 MHz RFR exposure (1 h/day, for 30 days, SAR: 0.9 W/kg) caused an increase oxidative stress and apoptosis in the thyroid gland and a decrease plasma nesfatin-1 level (Hussien et al., 2020). In a recent study of 15 normal-weight young men aged 21–29 years, it was stated that exposure to GSM 900 MHz RFR emitted by two different mobile phone types (phone 1: Motorola L2 (Motorola Mobility LLC), Libertyville, IL, USA and phone 2: Nokia 5800d-1 (Nokia Group, Helsinki, Finland), SAR: 0.97 W/kg for phone 2 and 1.33 W/kg for phone 1) for 25 min per day significantly increased calorie and food intake (Wardzinski et al., 2022). On the other hand, Tang et al. reported that nesfatin-1 had antioxidative and antiapoptotic effects on the brain, thus being neuroprotective. It was also observed that nesfatin-1 had a significant effect on the suppression of brain damage caused by oxidative mechanisms (Tang et al., 2012). Studies showed that nesfatin-1 is a

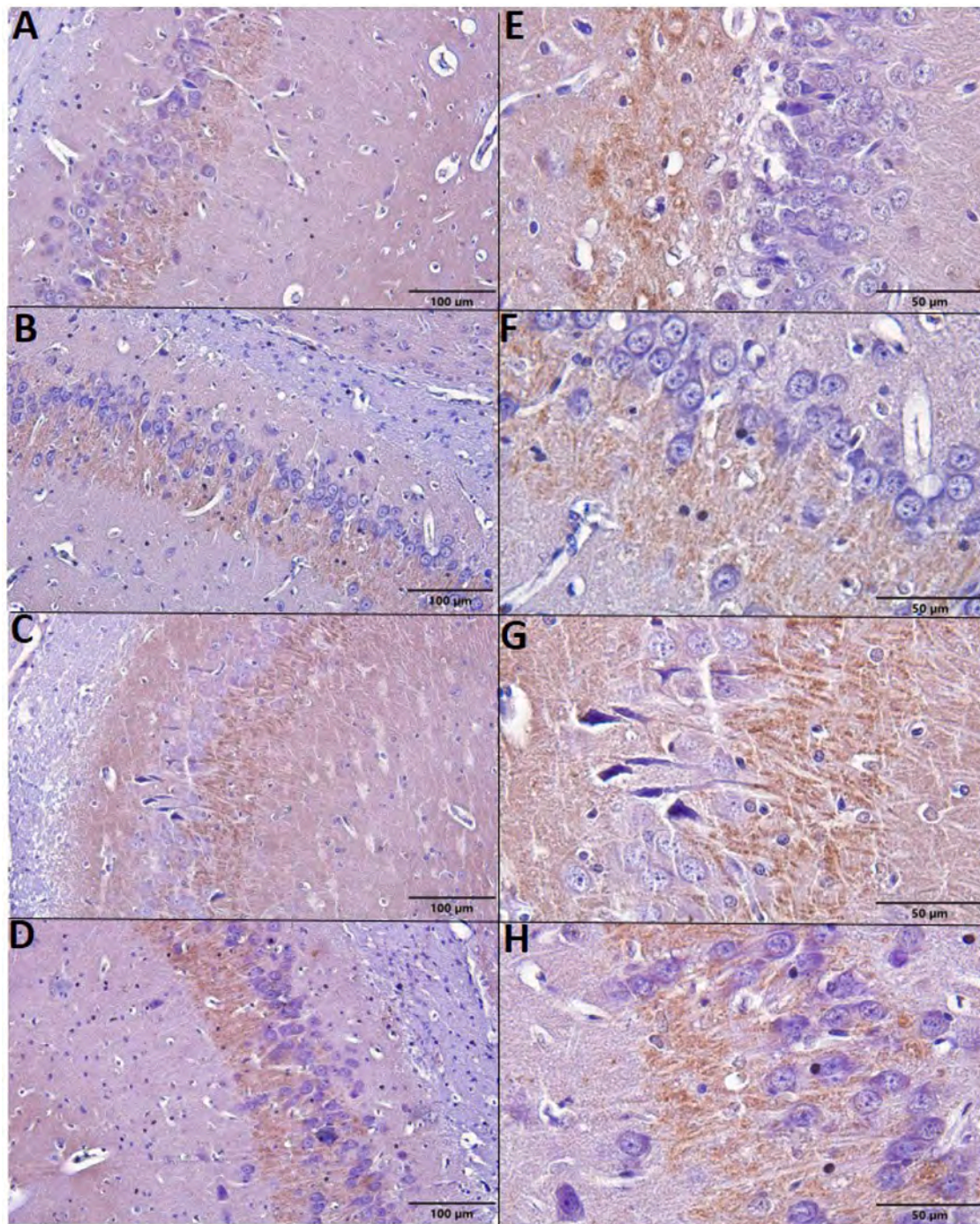


Fig. 4. Immunohistochemical images of anti-synaptophysin antibody in brain tissues of rats at 40 × and 100 × : A, E) Healthy sham (Group 1); B, F) Diabetes sham (Group 3); C, G) Healthy + 5 G (Group 2); D, H: Diabetes + 5 G (Group 4).

neuropeptide that plays an important role in stress responses and the pathology of mental and neurological disorders (Aydin et al., 2009; Kőnczöl et al., 2010; Palasz et al., 2012). In addition, it was found that ghrelin promoted the formation of new brain cells and protected them from the effects of aging (Morgan et al., 2018). There is also evidence that ghrelin enhances perception. Animals fed low-calorie diets were shown to experience an increase in mental abilities, and ghrelin was thought to be effective in that. Mice injected with ghrelin had improved performance in learning and memory tests. The findings suggested that ghrelin increases the neural connections in the brain (Morgan et al., 2018).

In our study, a significant decrease in irisin level was detected in the

brains of both diabetic and healthy rats exposed to RFR. Although irisin is a myokine secreted from skeletal muscles, it has major effects on various parts of the body, and particularly the central nervous system, impacting lipid metabolism, thermogenesis, browning of white adipose tissue, bone resorption, neuronal differentiation in the brain, and neuroprotection (Young et al., 2019). Moreover, it was stated that hippocampal neurogenesis is dose-dependently regulated by irisin (Moon et al., 2013). It was also reported that irisin expressed in the brain induces the expression of brain-derived neurotrophic factor in the rat hippocampus; thus, increased brain irisin levels can preserve memory. Moreover, it was shown in an Alzheimer rat model that an increase in irisin could prevent cognitive decline (Lourenco et al., 2019). On the

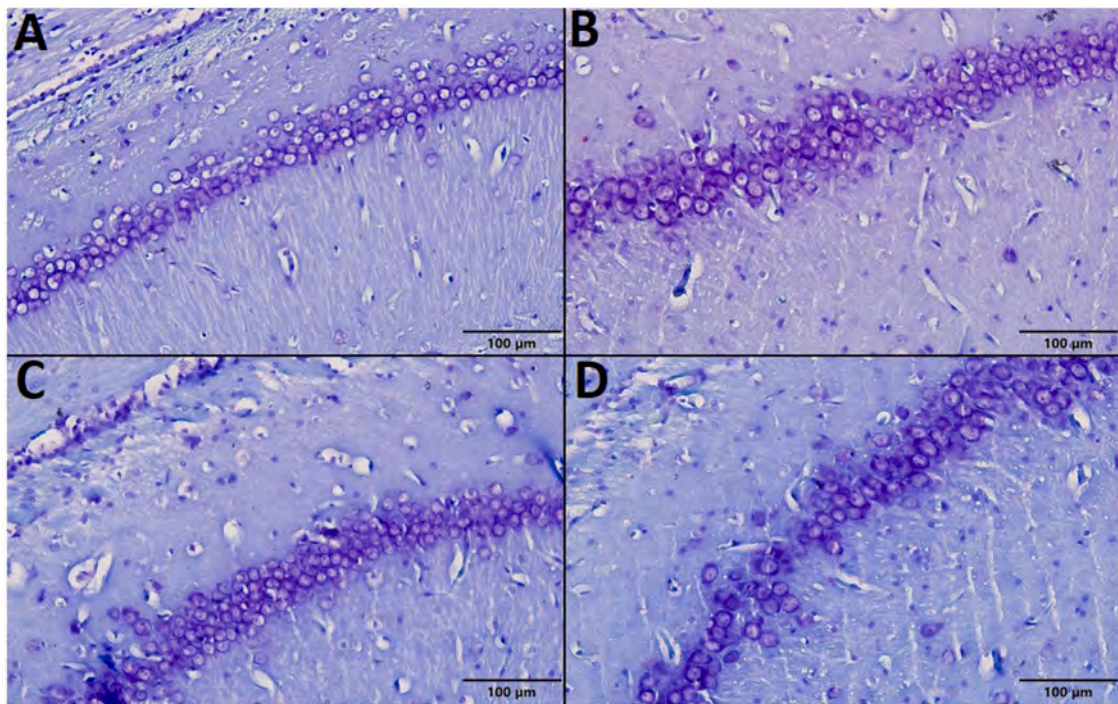


Fig. 5. Microscopic images of brain tissue stained with cresyl violet: A) Healthy sham (Group 1); B) Diabetes sham (Group 3), C) Healthy + 5 G (Group 2), D) Diabetes + 5 G (Group 4).

other hand, according to our results, as 5 G RFR causes a decrease in brain irisin levels, it can be thought that it may trigger neurodegenerative diseases such as Alzheimer disease.

According to our immunohistochemical results, no significant difference was observed between the groups in terms of synaptophysin (Fig. 1). Synaptophysin is a neuroendocrine tumor marker (Özaslan et al., 2014) and a synaptic protein involved in synaptic plasticity and cognitive function, and its amount is reduced in cases of synaptic degeneration induced by neuroinflammation (Yang et al., 2020; Zhou et al., 2020).

However, according to the cresyl violet staining results of the present study, the increase in degenerated neurons in the healthy and diabetic 5 G groups compared to the healthy and diabetic sham groups indicates that 3.5 GHz increases the number of degenerated neurons in the CA1 region of the hippocampus. This shows that long-term exposure to 3.5 GHz RFR may trigger dementia at later ages. It was also seen that 3.5 GHz RFR caused degeneration in the brain of healthy rats, and more intensely so in diabetic rats. Similarly, Hasan et al. performed cresyl violet staining in their study and reported that abnormally sized and shaped pyramidal cells were observed in the brains of mice exposed to RFR (4 G, 2400 MHz, 40–60 min/day for 60 days), and degenerated neurons were densely present (Hasan et al., 2022). There are many studies in the literature that provide evidence that RFR affects the central nervous system in the frequency range of 900–2400 MHz. It was reported that darkly stained granule cells in the DG areas of Wistar albino rats exposed to continuous modulated 900 MHz RFR (SAR: 2 W/kg) (Odaci et al., 2008), pyknotic neurons in the hippocampal CA region of Wistar albino rats exposed to 900 MHz RFR (Baş et al., 2013), and dark cytoplasm pyramidal neurons in the hippocampus of Sprague Dawley rats exposed to GSM 900 MHz RFR (SAR: 0.024 W/kg) (Şahin et al., 2015) were observed.

It was reported that GSM 900, 1800 MHz RFR exposure (for 60 min/5 days of the week during the one year SAR: from 8.6 to 93 mW/kg) increased the intracellular Ca^{2+} concentration and induced apoptosis in hippocampal and dorsal root ganglion neuron of Wistar rats (Ertilav et al., 2018). It is known that increased Ca^{2+} input will depolarize the

inner mitochondrial membrane by increasing electron transport chain activity and ROS production (Bektas, 2021). Moreover, ROS may be involved in the actions of RFR emitted from wireless communication devices on biological systems (Ilhan et al., 2004; Nazıroğlu et al., 2012).

5. Conclusion

The data obtained in this study showed that 3.5 GHz RFR caused changes in energy metabolism and appetite in both diabetic and healthy rats. In addition, it was shown that 3.5 GHz can cause degenerative effects via oxidative damage in the brain. It was demonstrated that these effects may be more dangerous for diabetics according to the TAS, TOS, H_2O_2 , ghrelin, nesfatin, and irisin levels and cresyl violet staining results. The results of this research reveal that 3.5 GHz RFR has the potential to alter nesfatin-1, irisin, and ghrelin levels. Further studies are needed.

Ethical statement

All experiments were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Experiments Local Ethics Committee of Van Yüzüncü Yıl University, Turkey (approval number: 2021/05-10). All experimental procedures were appropriate to the Guide for the Care and Use of Laboratory Animals of National Institutes of Health (NIH Publication No. 80-23; reformulated in 1996).

Author statement

All authors read and approved the final and revised manuscript. They warrant that the article is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere.

Authors' contributions

HB and **SD** designed the study. **HB** and **SA** performed the in vivo study. **HB** and **SA** performed the biochemical analysis. **FA** performed the histopathology analysis. **KY** performed SAR simulation. **HB**, **SD** and **ZA** wrote the manuscript, **SD** supervised all the step of the study, All authors read and approved the final manuscript.

Conflict of interests

The authors have no conflict of interest.

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Changes in the histopathology and in the proteins related to the MAPK pathway in the brains of rats exposed to pre and postnatal radiofrequency radiation over four generations

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ABSTRACT

The development of new technologies and industry increases the number and variety of electromagnetic field (EMF) sources. Researcher are increasingly interested in the effects of EMF on brain health. The brain's function is largely dependent on electrical excitability, so it would be expected to be vulnerable to EMF. We therefore investigated the effects of brain development in the fetus, histopathological changes in female rats and the hippocampal level of MAPK proteins in male rats after exposed to pre and postnatal 2450 MHz continuous wave (CW) radiofrequency radiation (RFR) over four generations. Four groups; sham, irradiated female, irradiated male, irradiated male and female, with each consisting of four rats (one male and three females) were created. Rats in the exposure groups were whole-body exposed to 2450 MHz CW-RFR for 12 h/day during the experiment. Irradiation started one month before fertilization in the experimental group. On the 18th day of the gestational period, one pregnant rat from each group was decapitated under general anesthesia and the fetuses were taken. The remaining two pregnant rats completed the normal gestation period. When the offspring were two months old, four rats, one male and three female, were allocated for the second generation study. Next generation animals were also experienced the same processes as the first generation rats. This study were evaluated development of brain in fetuses and histopathological changes in brain of female rats using haematoxylin eosin staining, and the hippocampal level of MAPK proteins in brain of male rats by Western Blotting. We observed hemorrhagic areas, irregular cellular localization and vascular structures in the brain of fetal and adult female rat of exposed groups in the all generations. pERK, ptau, pJNK and pp38 were increased in the brain of adult male rat of exposed groups in the all generations ($p < 0.005$). Pre and postnatal 2450 MHz continuous wave radiofrequency radiation exposure may cause changes in the function of the MAPK pathway affecting cognitive processes such as learning and memory and may cause damage to both the fetus and adult brain tissue. Also, EMF may have potential to affect brain of future generations.

1. Introduction

As a result of the rapid increase in wireless communication devices, such as mobile phones, base stations, Wi-Fi, bluetooth technologies, household appliances, radar devices, etc., electromagnetic pollution has become a concern for public health. Along with this increase, many in

vivo and in vitro research projects are still conflicted about risks to human health. In the last few decades, one of the most important research topics has been researching the effect of radiofrequency radiation (RFR) on the central nervous system. For instance, some researchers have indicated the effects of RFR on the function of brain, such as sleep, attention, learning, memory, and cognition (Hamblin and

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Wood (2002); Edelstyn and Oldershaw (2002); Koivisto et al., 2000; Nittby et al., 2008) and some of the researchers have indicated effects of RFR on the structure of the brain. For example, Dasdag et al. reported that “Long term RFR exposure has the potential to alter some bio-parameters, such as expression of microRNAs, protein carbonyl, oxidative stress, apoptosis, biochemical, and morphological changes in the brain. The results indicated that long term exposure to 2.4 GHz RF can cause harmful effects such as neurodegenerative diseases caused by the parameters stated above” (Dasdag et al., 2004, 2009, 2012, 2015, 2019; Akdag et al., 2010; Alkis et al., 2019).

Neurodegenerative diseases including Alzheimer’s disease (AD), Multiple Sclerosis (MS), Parkinson’s disease (PD), Amyotrophic Lateral Sclerosis (ALS), etc., are associated with neuronal loss and deposition of misfolded proteins in anatomic brain regions (McMackin et al., 2019). Animal studies showed that exposure to low-intensity EMF induced neurodegeneration. In addition, numerous epidemiological studies have shown a higher incidence of AD in human populations with higher EMF exposure (Pall, 2022).

Brain tissue consists of the neuronal cell body from the process (myelinated fiber or not, dendrites, and axons), interconnected glial cells, brain extracellular matrix, extracellular fluid, and blood vessels. Neuronal cells are the central nervous system (CNS)’s primary functional and structural units. Neurons function a using quick electrical impulse, which underly our opinions, behavior, and sensation of the world, so they would be expected to be vulnerable to EMF. It is known that abnormal brain electrical activity results in many brain disorders. RFR causes periodic alterations in neuronal electric parameters (Hinrikus et al., 2021).

The hippocampus is a part of the brain embedded deep in the temporal lobe of each cerebral cortex that controls some behavioral and cognitive functions, including spatial learning and working memory (Anand and Dhikav (2012)). It is affected by RFR (Lai, 2019). Many researchers have focused on behavioral problems caused by RFR exposure, including learning and memory loss in rats (Lai et al., 1994; Wang and Lai, 2000). Hasan et al. suggested that 2400 MHz RF-EMR cell phone radiation affects the structural integrity of the hippocampus, resulting in behavioral changes such as anxiety (Hasan et al., 2022). Shahin et al. showed that 2.45 GHz MW radiation induces oxidative and nitrosative stress in the hippocampus and impairs learning and spatial memory by causing apoptosis of neuronal and non-neuronal cells (Shahin et al., 2015). In another study, an association between a mother’s exposure to non-ionizing radiation of magnetic field during pregnancy and the risk of attention deficiency/hyperactivity disorder (ADHD) has been reported in animal and human studies. Li et al. suggested that in utero exposure to elevated levels of non-ionizing radiation is associated with an increased risk of especially ADHD with immune-related comorbidity (Li et al., 2020).

Tau is an intracellular soluble protein and it stabilizes microtubules in axons. It is also reported that “phosphorylation of Mitogen-Activated Protein Kinases (MAPK) plays a role in cells’ responses to stress and hyperphosphorylation, which may originate from an imbalance between the actions of protein kinases and protein phosphatases. Therefore, MAPKs may play a role as a key contributor in the neurodegeneration of Alzheimer’s disease” (Drewes et al., 1992; Lee et al., 2005).

Extracellular signal regulated kinases 1 and 2 (ERK1/2).

It is stated that “intracellular activation and extracellular signal regulated protein kinases 1 and 2 (ERK1/2), a MAPK subgroup of enzymes play a significant role in cell cycle progression, contributing to developmental brain activities. ERK1/2 is critical for neuronal differentiation and plasticity and may also modulate neuronal survival” (Hetman and Gozdz (2004); Alonso et al., 2004).

Some researchers defined that “the c-Jun NH2-terminal kinases (JNK) played an important role in cell regulation, contributing to functions, such as gene expression, cell proliferation, and programmed cell death as a member of the MAPK super-family (Kaneto et al., 2005; Abdelli et al., 2007; Weston and Davis (2007)) and JNK pathways are

closely associated with several diseases, including neurodegeneration, tumors, and diabetes” (Nateri et al., 2005; Tuncman et al., 2006; Wilhelm et al., 2007). However, the results of some experiments have indicated that “JNK signaling plays a key role an important role in tau protein phosphorylation in neuronal tissues” (Reynolds et al., 1997; Yoshida et al., 2004; Schechter et al., 2005). It was stated that “the p38 MAPK family plays an important role in tau protein phosphorylation” (Li et al., 2003; Mendonsa et al., 2009).

Neuronal damage to the central nervous system due to electromagnetic radiation has been reported in both prenatal and early adults (Mausset et al., 2001; Salford et al. 2003). Despite the results of many experimental studies performed on RFR and the brain, it is stated that “the potential effects of EMF exposure on the central nervous system are still unclear” (Lazzaro et al., 2013; Altunkaynak et al., 2016).

Due to uncertainties on the subject and insufficient generation studies, this study aimed to investigate the effects of pre-and postnatal 2450 MHz CW-RFR exposure on brain development, histology, and the hippocampal MAPK proteins in four generations of rats.

2. Materials and methods

2.1. Experimental animals

All tentative protocols applied to the rats were conducted following the Principles of Laboratory Animal Care and the rules of the Scientific and Ethics Committee of Experimental and Clinical Research Center of Erciyes University. This research was confirmed by the Local Ethics Committee of Animal Experiments of Erciyes University (Decision No: 16/111). Twelve female and four male Wistar Albino rats were used in this study at the beginning. All animals were obtained and maintained from Erciyes University Experimental Research and Application Center (Kayseri, Turkey). Animals were housed under standard conditions (12 h light / 12 h dark cycle and the temperature was kept at 23 ± 2.5 °C). They were fed ad libitum, and tap water was provided as drinking water. Water and food were available from this center. The rats were divided into sham control and three exposure groups, with one male and three female rats in each group (Fig. 1). During the experiment, rats in the exposure groups were whole-body exposed to 2450 MHz CW-RFR for 12 h/day. In the first exposure group, only male rats were exposed. Both female and male rats were exposed in the second exposure group, and only female rats were exposed in the third group. The same procedure was applied to the sham control group rats, except for the generator being turned off.

2.2. Radiation exposure

This study used an RFR generator (220 V; 2370–2450 MHz, 0–4 Watt; Nar Technology, Kayseri, Turkey) with a 2450 MHz continuous wave to expose the animals. The study was performed in a Faraday cage to eliminate environmental exposures sourced from mobile phones, base stations, and radio-TV transmitting stations. A cylindrical cage with wire grid top and bottom covers enabled free-air access to the rats during exposure measurements. Wire-grid spacing was selected as 5 mm, which was much smaller than free-space wavelength λ_0 , at 2450 MHz. Experimental and simulation setups are illustrated in Fig. 2. Before RFR exposure, ambient RF level was measured with a wide band antenna (Log-periodic Antenna, HF59B, FF6E, Gigahertz Solutions, Langenzenn, Germany) on the inner and outer surface of the Faraday cage. The power of the RF generator was set to 1 Watt. RF field levels inside and outside the Faraday cage were remeasured with the generator turned on. The electromagnetic power density was recorded as 8.5 ± 0.65 W/m² inside each section of the Faraday cage.

Rats could freely move in the cage. The entire study was conducted at the Erciyes University Research Center in a room (Temperature: 25 ± 2 °C, Humidity: 40%–50%, 12 h constant light-dark cycle) allocated explicitly for this research. Animals were fed ad libitum, and tap water

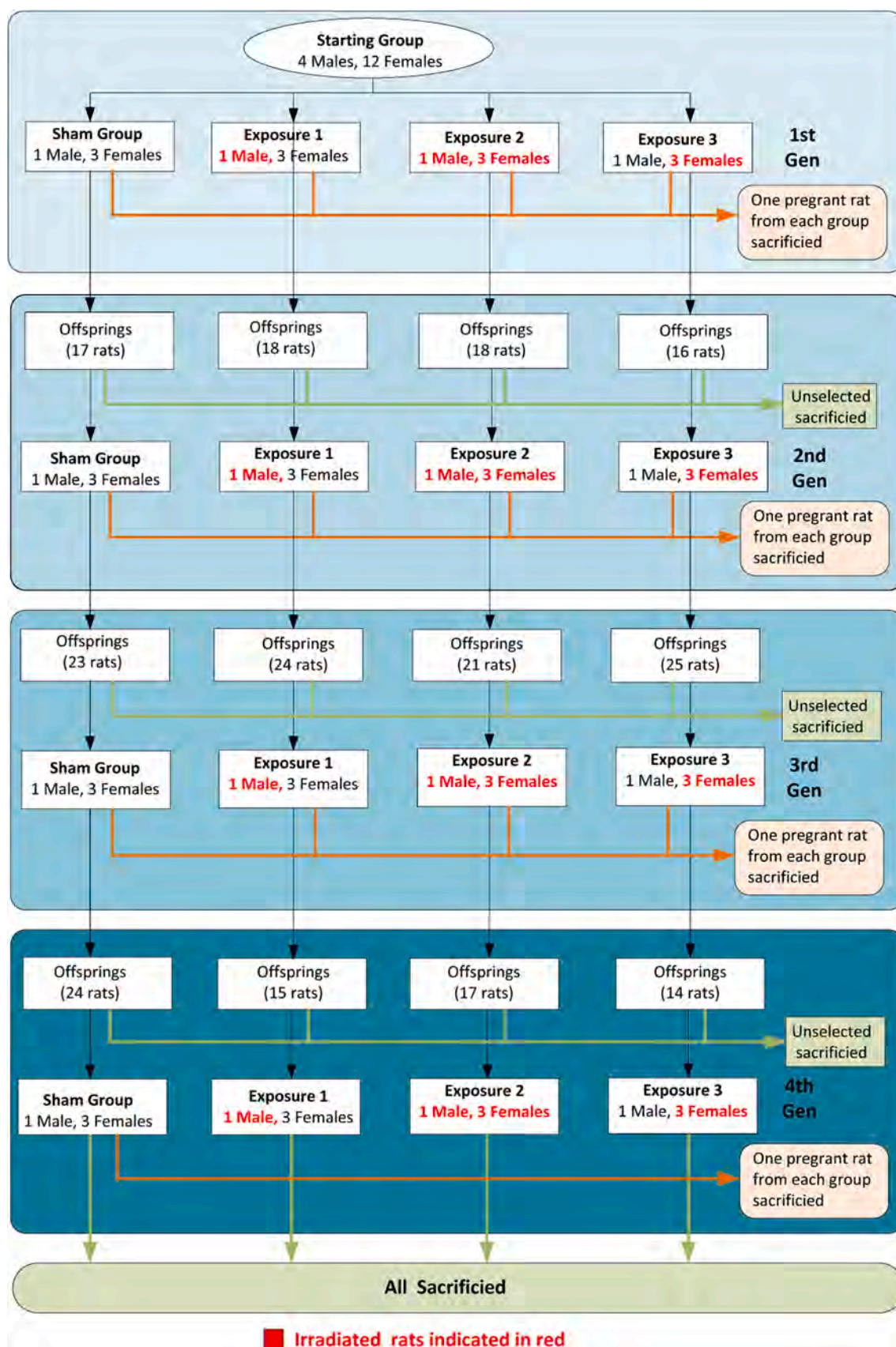


Fig. 1. Rats in respective groups and generations.

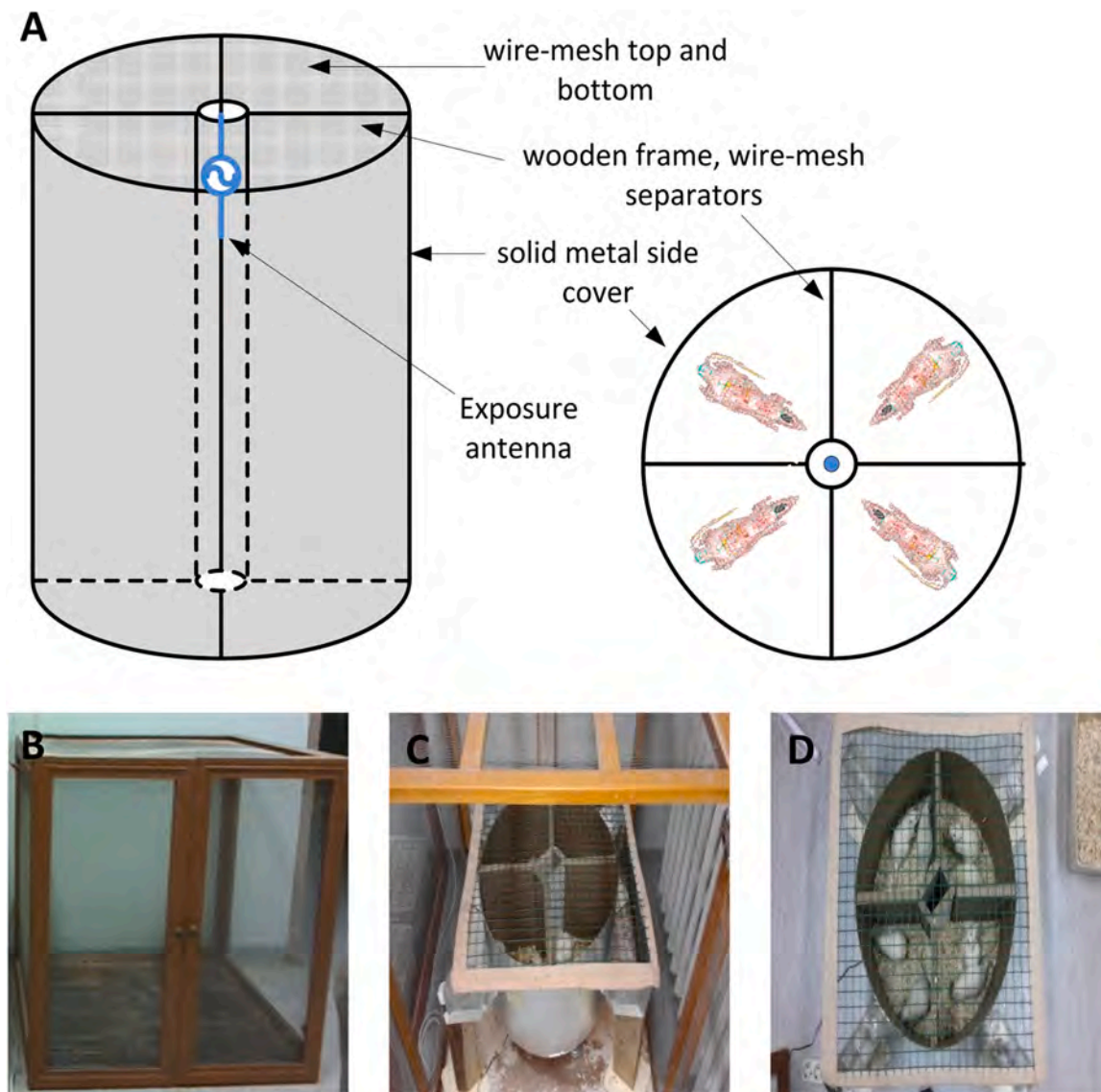


Fig. 2. Simulation and experimental setups. (a) Large circular cage to house rats and for RF irradiation. (b) Large rectangular Faraday cage. (c) Circular cage inside the rectangular Faraday cage (d) Top view of the Faraday cage and circular cage.

was provided as drinking water. Experimental protocols and study design are illustrated in Fig. 3. All the animals were exposed to RFR between 08:00 and 20:00. RF exposure started one month before fertilization, and fertilization took place at the end of this period (first generation). A vaginal smear test determined pregnancy. One pregnant rat from each group was decapitated by intraperitoneal xylazine (mg/kg) and ketamine (mg/kg) injection on the 18th day of the gestational period, and the fetuses were taken.

The remaining two pregnant rats completed the normal gestation period (21 days). When the offspring were two months old (second generation), four rats, one male, and three females, were allocated for the next-generation study. The remaining offspring were sacrificed under general anesthesia, and their organs were removed. One second-generation pregnant rat from each group was decapitated by intraperitoneal xylazine (mg/kg) and ketamine (mg/kg) injection on the 18th day of the gestational period, and the fetuses were taken. The remaining two pregnant rats completed the normal gestation period. When the offspring were two months old (third-generation), four rats, one male, and three females, were allocated for the next-generation study. The remaining offspring were sacrificed under general anesthesia, and their organs were removed. One third-generation pregnant rat from each

group was decapitated by intraperitoneal xylazine (mg/kg) and ketamine (mg/kg) injection on the 18th day of the gestational period, and the fetuses were taken. The remaining two pregnant rats completed the normal gestation period. When the offspring were two months old (fourth generation), four rats, one male, and three females, were allocated for the next-generation study. The remaining offspring were sacrificed under general anesthesia, and their organs were removed. One fourth-generation pregnant rat from each group was decapitated by intraperitoneal xylazine (mg/kg) and ketamine (mg/kg) injection on the 18th day of the gestational period, and the fetuses were taken. The remaining two pregnant rats completed the normal gestation period. When the offspring were two months old, all rats were decapitated under general anesthesia to remove their organs.

In summary, the irradiation started one month before the pregnancy of the first generation and continued until the fourth-generation animals were sacrificed. However, successive generation animals were also designed and experienced the same processes as the first-generation rats.

2.3. Specific absorption rate (SAR)

The measurement setup consisted of the applicator antenna and the

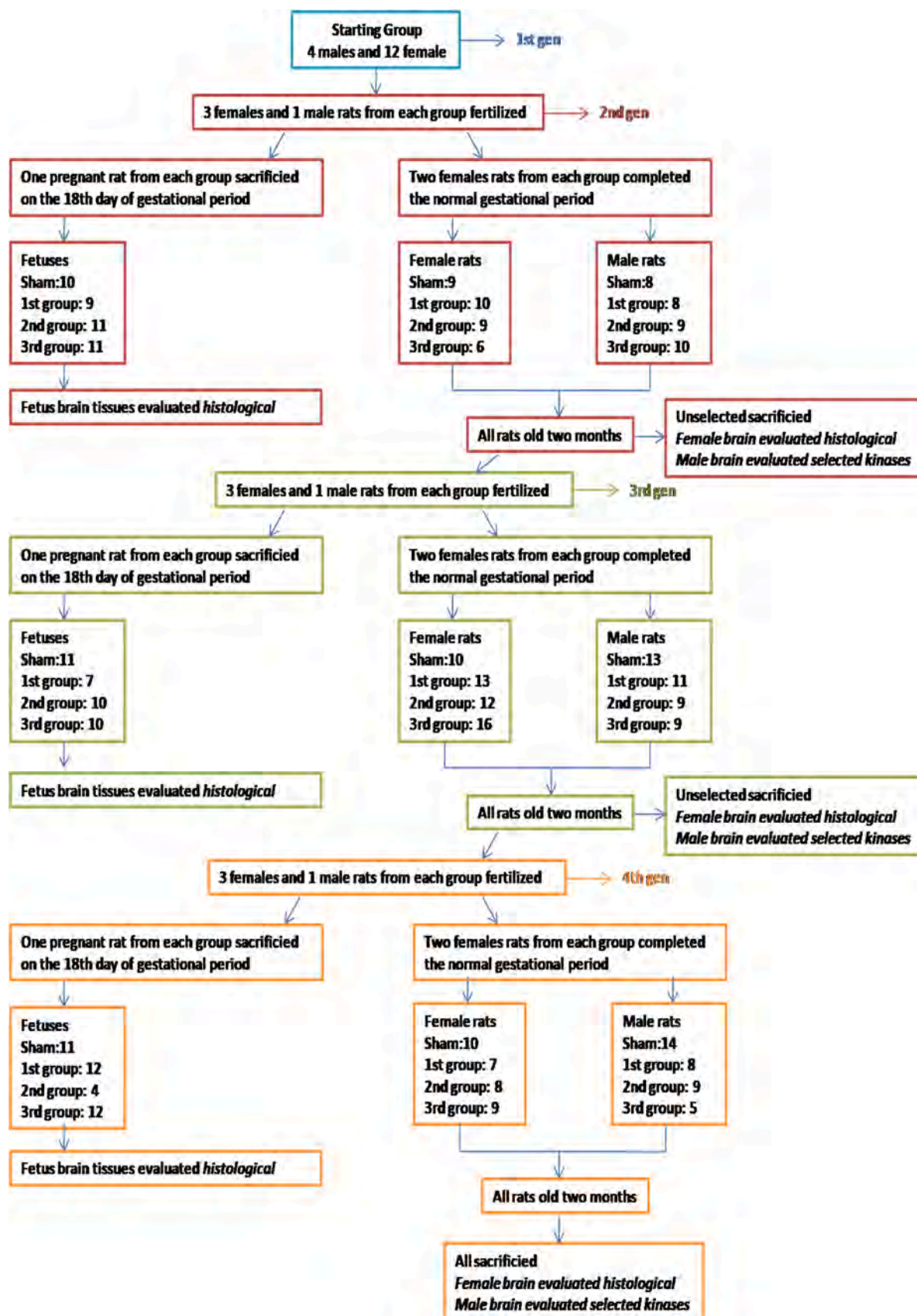


Fig. 3. Experimental protocols and study design.

Faraday cage, and the rats were modeled in a 3D electromagnetic field solver using CST (CST AG, Darmstadt, Germany). Electromagnetic and thermal modeling within the CST is based on finite-integration technique, a combined method of integral and differential solvers acting on space and time, respectively. Using an IEEE/IEC 62704-1 method, whole-body SARs were calculated as 0.186 W/kg for 1 g average and 0.093 W/kg for 10 g average. SAR in the brain was calculated as 0.071 W/kg and 0.118 W/kg for 10 g and 1 g average, respectively. Figures display the rat voxel model and SAR distribution for 10 g and 1 g averages, Figs. 4A, 4B, and 4 C, respectively. The antenna and the rats were symmetrically placed in the simulation setup to yield no variation from one chamber to another. However, SAR levels can change slightly due to size differences between the animals. This variation is expected to be small as the mass of rats does not differ more than 5% of the mean mass in exposure groups. To simulate SAR for offspring, another 3D electromagnetic field simulator, Sim4Life (ZMT Zurich MedTech AG, Zurich, Switzerland), was used because only Sim4Life has a 7-day old

infant rat voxel model that could be used for SAR simulations. Sim4Life utilizes a finite difference time domain technique as a 3D electromagnetic field solver. A similar exposure setup was replicated in Sim4Life, and SAR in the brain was calculated as 0.204 W/kg for 1 g average. Whole-body SAR for offspring was calculated as 0.287 W/kg. Whole-body SAR and SAR in the brain for offspring were 1.54 and 1.74 times more than the adults, respectively.

2.4. Histopathological evaluation

Fetuses and adult female rats' brain tissues were excised from each animal, and then the tissues were cut longitudinally. The fetuses' numbers were 41, 38, and 39, respectively in the 2nd, 3rd, and 4th generations (Fig. 3). The adult female rats' numbers were 34, 51, and 34, respectively in the 2nd, 3rd, and 4th generations (Fig. 3).

The tissues were fixed in 10% formaldehyde (Merck, Darmstadt, Germany) for 24 h. Specimens were dehydrated through graded

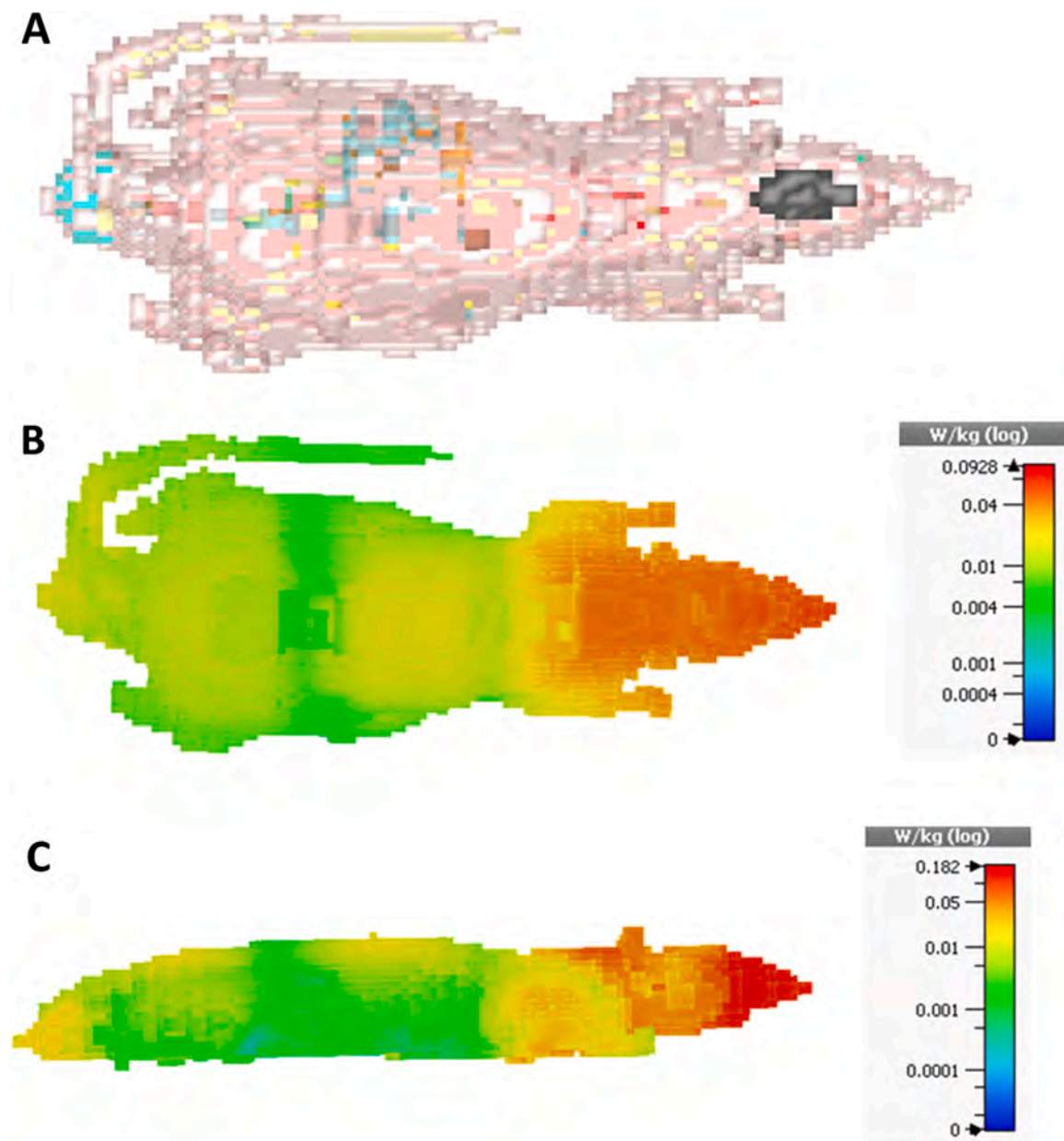


Fig. 4. SAR simulations at 2450 MHz. (a) Adult rat voxel model. (b) Adult rat sagittal cut whole body SAR distribution for 1 g average. (c) One week old baby rat model.

concentrations of ethanol (70%, 80%, 96%, 100%, Merck, Darmstadt, Germany), cleared with xylene (Merck, Darmstadt, Germany) and embedded in paraffin wax (Leica, Buffalo Grove, US); 5 µm thick sections were cut from each paraffin block. Then, sections were stained with haematoxylin and eosin (Thermo Scientific, Runcorn, UK) to show histological changes. After staining, the sections were observed using a light microscope (Olympus BX51, Tokyo, Japan) and images were captured.

2.5. Western blot

The adult male rats' numbers were 35, 42, and 36, respectively in the 2nd, 3rd, and 4th generations (Fig. 3). For the Western blot studies, the adult male rats were decapitated after the end of the appropriate applications and their hippocampi were dissected immediately. After isolation of the protein with RIPA lysis buffer (sc-24948; Santa Cruz Biotechnology, California, USA) from the hippocampal tissues, the amount of protein was determined by the Bicinchoninic Acid (BCA Protein Assay Reagent A, Thermo scientific Prod #23223 and BCA Protein Assay Reagent B 25, Thermo scientific Prod #23224) and loaded into the SDS-PAGE gel (Mini-PROTEAN, Bio-rad) with 20 µg protein per well. Proteins were run with SDS-PAGE gel, with maximum voltage (V) and 25 milliamperes (mA) per gel, and transferred to the PVDF membrane (10600021, GE Healthcare Life Science, Amersham Hybond P 0.2 PVDF 260 mm × 4 m, Marlborough, USA). After one hour of blocking with 5% non-fat milk powder (Sigma Aldrich, 70166), samples were prepared in TBS-T (10 mM TRIS-HCl pH 7.4, 150 mMNaCl, 0.1% Tween 20), and the blots were incubated overnight with primary antibodies (anti-P38 MAPK, 1:1000; anti-p-P38 MAPK, 1:1000; anti-ERK1/2 (p42/44 MAPK), 1:1000; anti-p-ERK1/2 (p-p42/44 MAPK), 1:1000; anti-JNK, 1:1000; anti-p-JNK, 1:1000; anti-Tau, 1:1000; anti-p-Tau (ser416), 1:500; anti-p-Tau (Thr231), 1:1000; anti-β-Actin, 1:1000) at + 4 °C. After incubated with primary antibody, the membranes were washed with TBS-T and treated with HRP-conjugated secondary antibodies (5% in milk powder, anti-mouse and anti-rabbit, 1–5000) for one hour at room temperature. Imaging was performed on the G: Box Syngene device (Beacon House, Cambridge, UK) to receive a signal with the ECL solution. According to the manufacturer's instructions, the bound antibodies were visualized using an ECL substrate solution (Cat: 32106 Thermo Scientific, Waltham, USA). The blots were subsequently stripped and reprobed with a β-actin mouse monoclonal antibody to confirm the gel's equal loading of protein samples. The optical density of the blots was measured with ImageJ software (National Institutes of Health, USA), was normalized to the expression levels of β, which was expressed as an arbitrary unit relative to a sample of the control group in each membrane. The list of primary and secondary antibodies is given in Table 1.

2.6. Statistical analysis

All calculations were made using IBM SPSS Statistics 20.0 software

Table 1
The list of primary and secondary antibodies using in the Western Blot.

Antibody	Host	Supplier/Catalog number	Dilution
Tau (Tau46)	Rabbit	Cell Signaling/#4019	1:1000
pTau ^{Ser416} (D7U2P)	Rabbit	Cell Signaling/#15013	1:1000
pTau ^{Thr231}	Mouse	Santa Cruz/sc-32276	1:500
ERK1/2	Rabbit	Cell Signaling/#9102	1:1000
p-ERK1/2	Rabbit	Cell Signaling/#9101	1:1000
JNK	Rabbit	Cell Signaling/#9252	1:1000
p-JNK	Rabbit	Cell Signaling/#9251	1:500
P38MAPK	Rabbit	Cell Signaling/#9212	1:500
p-P38MAPK	Rabbit	Cell Signaling/#9211	1:500
β-Actin	Mouse	Santa Cruz/sc-69879	1:500
Anti-rabbitIgG-HRP	Goat	Santa Cruz/sc-2004	1:4000
anti-mouse IgGK BP-HRP	Mouse	Santa Cruz/sc-516102	1:4000

(IBM, ILL, USA). Data were evaluated for normality and sphericity using the Shapiro-Wilks and Mauchly's test. These tests confirmed that the data showed normal distribution and sphericity. Shapiro-Wilks and Levene tests were performed for normality and homogeneity. These tests confirmed that the data had equal variance. Statistical comparisons were evaluated by one-way ANOVA followed by an LSD test. Statistical significance was accepted at $P < 0.05$.

3. Results

No, differences were recorded in the gross anatomy of all generation rats.

3.1. Fetal brain tissue findings

When we investigated histopathological changes in the fetuses belonging to second-generation rats exposed to 2450 MHz CW-RFR, it was observed that fetuses' brain tissues belonging to the sham control group had normal histological structure when evaluated under a light microscope. The brain tissue was surrounded by a prominent pia mater in this group. The cells forming the cortex showed a regular stratification. It was noted that among the groups exposed to EMF, the presence of hemorrhagic areas and the irregular cellular localization in the cortex, especially in 1st and 2nd groups compared to the sham control group. It was observed that these histopathological findings were more minor in the 3rd group compared to the 1st and 2nd groups (Fig. 5).

Also, in histopathological changes in the fetuses belonging to third- and fourth-generation rats, the brain tissue was surrounded by the pia mater, the inner most layer of the meninges. At this stage of development, the cortex and medulla layers could be separated. In haematoxylin-eosin stained sections, neurons were distinctly distinguished. Like adult female brain tissue, vascular structures were taken attention in this stage of development in experimental groups (Fig. 5). There was also no distinct difference between generations.

3.2. Adult female brain tissue findings

Regarding histopathological changes in the brain belonging to second-generation female rats exposed to EMF, it was observed that adult female brain tissues belonging to the sham control group had normal histological structure when evaluated under a light microscope. The brain tissue was surrounded by a prominent pia mater in this group. The cells forming the cortex showed a regular stratification. It was noted that among the groups exposed to EMF, the presence of hemorrhagic areas and the irregular cellular localization in the cortex, especially in the 1st and 2nd groups, compared to the sham control group. It was observed that these histopathological findings were more minor in the 3rd group compared to the 1st and 2nd groups (Fig. 6).

Regarding histopathological changes in the brain belonging to third- and fourth-generation female rats, the cerebral cortex was externally surrounded by pia mater, a loose connective tissue. In the sham control group, neurons and neuroglia cells with well-preserved cytoplasm and nucleus were observed in the cerebral cortex and normal histological structure. When the experimental groups were compared to the sham control group, vascular dilatation areas and cellular localization irregularity were observed in some cortex regions, especially in groups 1 and 2 exposed to EMF. In addition, there were no significant histological differences between generations (Fig. 6).

3.3. Adult male brain tissue findings

To investigate whether the phosphorylation status of Tau and MAPKs correlated with exposure to EMF, we measured the levels of total and phosphorylated forms of Tau and MAPKs protein (Figs. 7, 8 and 9) by Western blotting assay.

For the second-generation male rats, it is shown in Fig. 7 that there

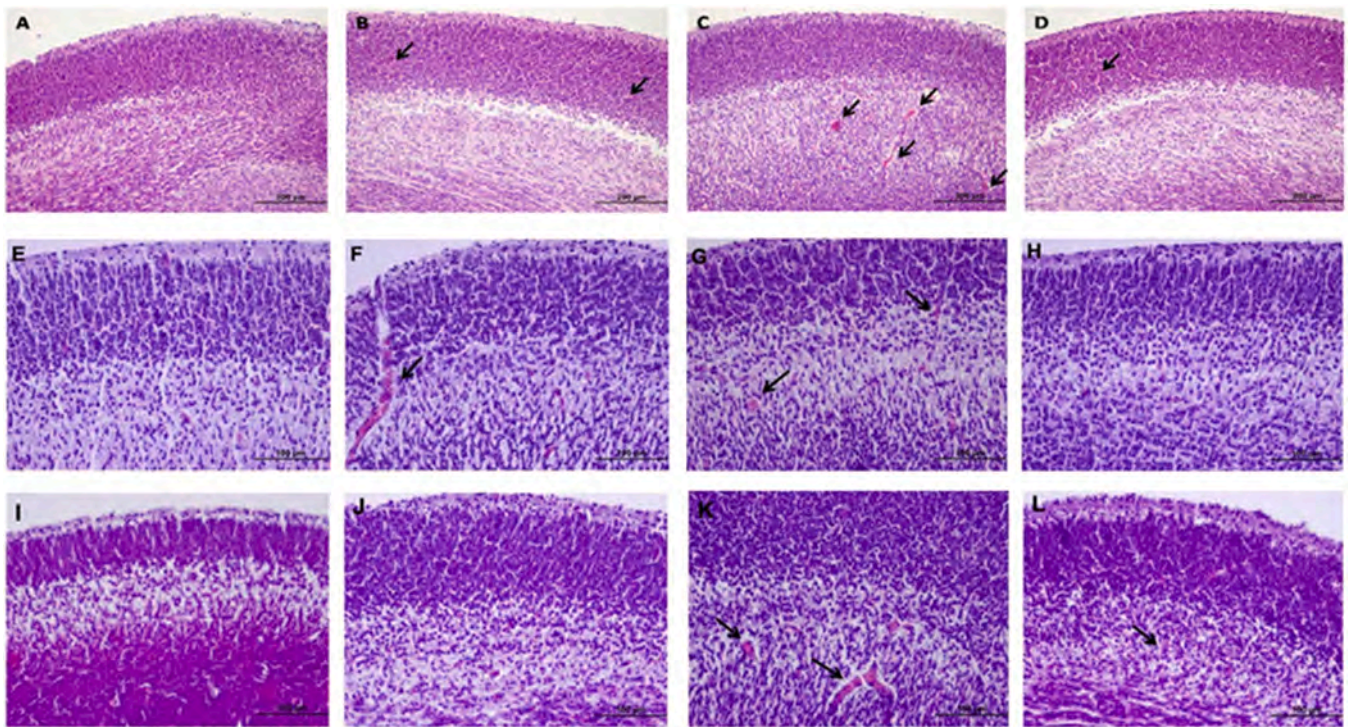


Fig. 5. Histopathological changes in the fetuses belonging to all generation rats. Fetuses brain tissues belonging to the sham control group had normal histological structure. The brain tissue was surrounded by a prominent pia mater. The cells forming the cortex showed a regular stratification. Neurons were distinctly distinguished. In the fetuses belonging to second generation rats, the exposure groups had hemorrhagic areas and the irregular cellular localization in the cortex, especially in first and second groups compared to the sham control group. (a) Sham control group. (b) First group. (c) Second group. (d) Third group. (Arrow; hemorrhage area). In the fetuses belonging to third generation rats, vascular structures were taken attention in this stage of development in exposure groups. (e) Sham control group. (f) First group. (g) Second group. (h) Third group. (Arrow; blood vessel). In the fetuses belonging to fourth generation rats, vascular structures were taken attention in this stage of development in exposure groups. (i) Sham control group. (j) First group. (k) Second group. (l) Third group. (Arrow; blood vessel; H&E staining x400, Olympus, BX51, Tokyo, Japan).

were significant differences between the sham control group and exposure groups in pERK and p-Tau (Ser416) ($p < 0.05$). However, there was no statistically significant difference between the sham control and exposure groups in total-Tau, total-ERK, total-p38 and p-P38 levels ($p > 0.05$). The p-ERK level was significantly increased in the hippocampus of the 1st group ($p = 0.035$) compared to the sham control group, and no significant difference was found in the 2nd and 3rd groups. In addition, the p-Tau (Ser 416) level of the 3rd group ($p = 0.045$) was significantly increased in the hippocampus of the rats compared to the sham control group. Still no significant difference was found in the 1st and 2nd groups compared to that sham control group.

We further analyzed the phospho/total protein ratio of Tau and MAPKS to obtain indirect information on activity levels. For the second generation male rats, it is shown in Fig. 7c that there were significant differences between the sham control group and exposure groups in p-ERK/t-ERK and p-Tau(Ser416)/t-Tau ratios ($p < 0.05$). However, there was no statistically significant difference between the sham control and exposure groups in the p-P38/t-P38 ratio ($p > 0.05$). The p-ERK/t-ERK ratio was significantly increased in the hippocampus of the 1st group ($p = 0.025$) compared to that the sham control group, and no significant difference was found in the 2nd and 3rd groups. In addition, the p-Tau (Ser 416)/t-Tau ratio of the 3rd group ($p = 0.042$) was significantly increased in the hippocampus of the rats compared to that sham control group. Still no significant difference was found in the 1st and 2nd groups compared to the sham control group.

For the third-generation male rats, Fig. 8 shows there were significant differences between the sham control group and exposure groups in Total-Tau, p-ERK, p-P38 and p-JNK ($p = 0.028$; 0.022 ; 0.007 ; 0.027 ; respectively). The p-ERK level was significantly higher in the hippocampus of the 1st ($p = 0.040$) and 2nd ($p = 0.008$) groups than in that

the sham control group, and no significant difference was found in the 3rd group. The p-JNK level was significantly increased in the hippocampus of the 1st group ($p = 0.013$) compared to that sham control group, but no difference was found in the 2nd and 3rd groups ($p > 0.05$). The p-P38 was significantly increased in the hippocampus of the 2nd group ($p = 0.002$) compared to sham control group, but no difference was found in the 1st and 3rd groups ($p > 0.05$). Total-Tau level was significantly increased in the hippocampus of the 2nd group ($p = 0.013$) compared to that sham control group. Still, no significant difference was found in the other groups compared to the sham control group. In addition, the p-Tau (Ser416) level was significantly increased in the hippocampus of the 1st group ($p = 0.043$) compared to that sham control group, but no significant difference was found in the other groups compared to the sham control group.

For the third-generation male rats, it is shown in Fig. 8c, that there were significant differences between the sham control group and exposure groups in p-Tau (Ser416)/t-Tau, p-ERK/t-ERK, p-P38/t-P38 and p-JNK/t-JNK ($p < 0.05$). The p-ERK/t-ERK ratio was significantly higher in the hippocampus of the 1st ($p = 0.021$) group than in that sham control group, and no significant difference was found in the 2nd and 3rd groups. The p-JNK level was significantly increased in the hippocampus of the 1st group ($p = 0.007$) compared to that sham control group, but no difference was found in the 2nd and 3rd groups ($p > 0.05$). p-P38 was significantly increased in the hippocampus of the 2nd group ($p = 0.003$) compared to sham control group, but no difference was found in the 1st and 3rd groups ($p > 0.05$). The p-Tau/t-Tau ratio was significantly increased in the hippocampus of the 1st group ($p = 0.033$) compared to that sham control group, but no significant difference was found in the 2nd and 3rd groups compared to the sham control group.

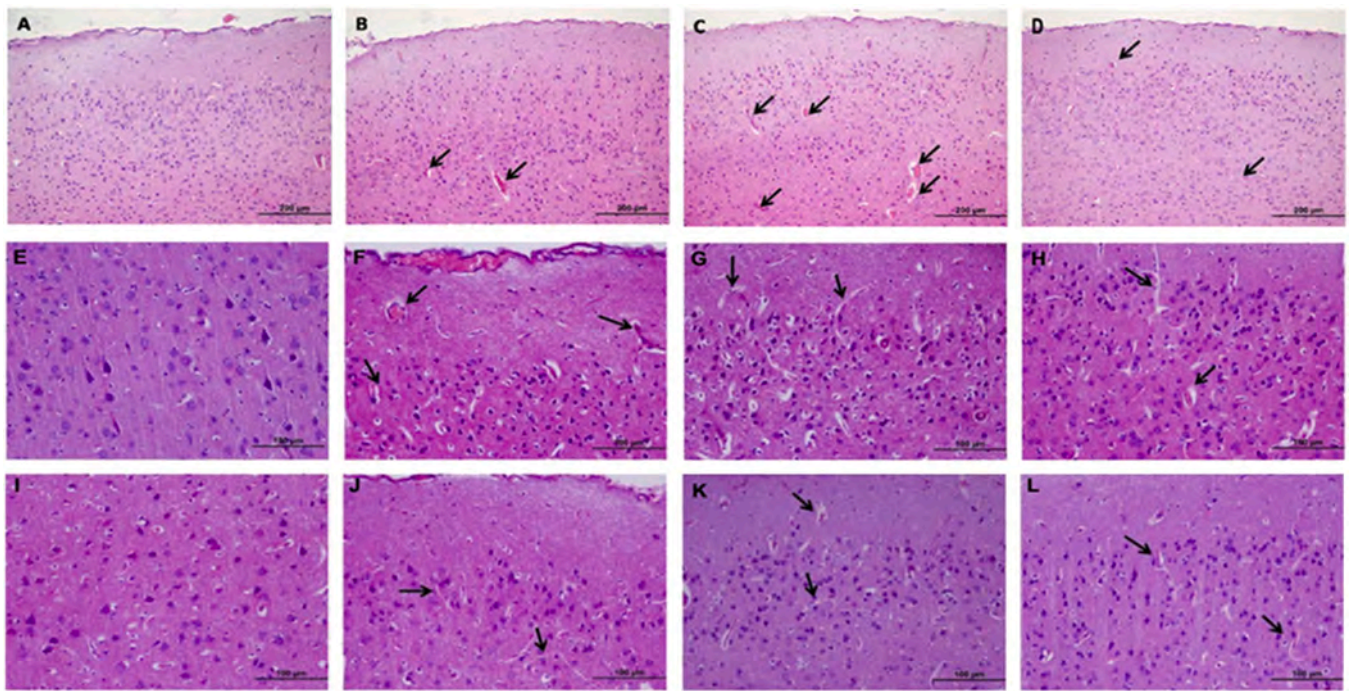


Fig. 6. Histopathological changes in the brain belonging to all generation adult female rats. The sham control group had normal histological structure. The brain tissue was surrounded by a prominent piamater, a loose connective tissue. The cells forming the cortex showed a regular stratification. In the sham control group, neurons and neuroglia cells with well-preserved cytoplasm and nucleus were observed in the cerebral cortex and they had normal histological structure. The cerebral cortex was externally surrounded by pia mater, a loose connective tissue. In the adult female rats belonging to second generation rats, exposure groups had hemorrhagic areas and the irregular cellular localization in the cortex, especially in first and second groups compared to the sham control group. These histopathological findings were less in the third group compared to the first and second groups. (a) Sham control group. (b) First group. (c) Second group. (d) Third group. In the brain belonging to third generation adult female rats, exposure groups had vascular dilatation areas and cellular localization irregularity were observed in some regions of the cortex especially in first and second groups. (e) Sham control group. (f) First group. (g) Second group. (h) Third group. In the brain belonging to fourth generation adult female rats, exposure groups had vascular dilatation areas and cellular localization irregularity were observed in some regions of the cortex especially in 1st and 2nd groups. (i) Sham control group. (j) First group. (k) Second group. (l) Third group. (Arrow; blood vessel H & E staining x 400, Olympus, BX51, Tokyo, Japan).

For the fourth-generation male rats, it is shown in Fig. 9 that there were significant differences between the sham control group and exposure groups in p-Tau (Ser416). However, no significant difference was found between the groups regarding in terms of total and phosphorylation levels of MAPKs and Total-Tau levels. The p-Tau (Ser416) was significantly increased in the hippocampus of the 1st ($p = 0.005$) and 3rd ($p = 0.042$) groups compared to that sham control group, but no difference was found in the 2nd group ($p > 0.05$). The p-ERK was significantly increased in the hippocampus of the 2nd group ($p = 0.036$) compared to the sham control group, but no difference was found in the 1st and 2nd groups ($p > 0.05$). p-JNK was significantly increased in the hippocampus of the 1st ($p = 0.046$) and 2nd ($p = 0.048$) groups compared to the sham control group, but no difference was found in the 3rd group ($p > 0.05$). p-P38 was significantly increased in the hippocampus of the 1st ($p = 0.041$) and 2nd ($p = 0.038$) groups compared to the sham control group, but no difference was found in the 3rd group ($p > 0.05$).

For the fourth-generation male rats, it is shown in Fig. 9c that there were significant differences between the sham control and exposure groups in the p-P38/t-P38 ratio ($p < 0.05$). However, no significant difference was found between the groups in p-Tau (Ser416)/t-Tau, p-Tau(Thr231)/t-Tau, p-ERK/t-ERK, and p-JNK/t-JNK ($p < 0.05$). The p-P38/t-P38 ratio was significantly increased in the hippocampus of the 1st, 2nd, and 3rd groups ($p = 0.020$; 0.001 ; 0.001 ; respectively) compared to the sham control group, but no difference was found in the 1st and 2nd groups ($p > 0.05$). p-JNK was significantly increased in the hippocampus of the 1st ($p = 0.046$) and 2nd ($p = 0.048$) groups compared to the sham control group, but no difference was found in 3rd group ($p > 0.05$). p-P38 was significantly increased in the hippocampus of the 1st ($p = 0.041$) and 2nd ($p = 0.038$) groups compared to the sham

control group, but no difference was found in the 3rd group ($p > 0.05$).

A 2×2 ANOVA was used to evaluate the differences between generations. A 2×2 ANOVA revealed that there was a significant generation effect ($F_{1,24} = 8.649$; $p = 0.007$), and non-significant group effect ($F_{3,24} = 2.734$; $p = 0.066$) and generation X group interaction ($F_{3,24} = 2.059$; $p = 0.132$) for pTau (Ser416). Post-hoc Tukey test showed that phosphorylation of Tau at Ser416 residue increased in third generation rats than second generation ($p = 0.048$). There wasn't significant difference between the second and fourth generation ($p > 0.05$) and between the third and fourth generation ($p > 0.05$). There was also a significant generation effect ($F_{1,24} = 8.544$; $p = 0.007$), and non-significant group effect ($F_{3,24} = 1.156$; $p = 0.347$) and generation X group interaction ($F_{3,24} = 0.987$; $p = 0.416$). No significant generation effects were found for total-Tau, pTau (Thr231), total-ERK1/2, pERK1/2, total-P38, total-JNK, and pJNK ($F_{1,24} = 2.757$; $p = 0.110$). Post-hoc Tukey test showed that phosphorylation of P38 increased in fourth generation rats than second generation ($p = 0.048$). There wasn't significant difference between the second and third generation ($p > 0.05$) and between the third and fourth generation ($p > 0.05$).

4. Discussion

4.1. Fetal brain tissue

Knowing whether or how sensitive the brain to RFRs in the developing embryo and childhood is vital in understanding the biological effects of electromagnetic pollution. Early fetal stages throughout childhood are an overly sensitive process, and the role of pre- and post natal RFR exposure is not sufficiently clear. It is stated that "neurons in most areas of the brain are formed during gestation and the neurogenesis

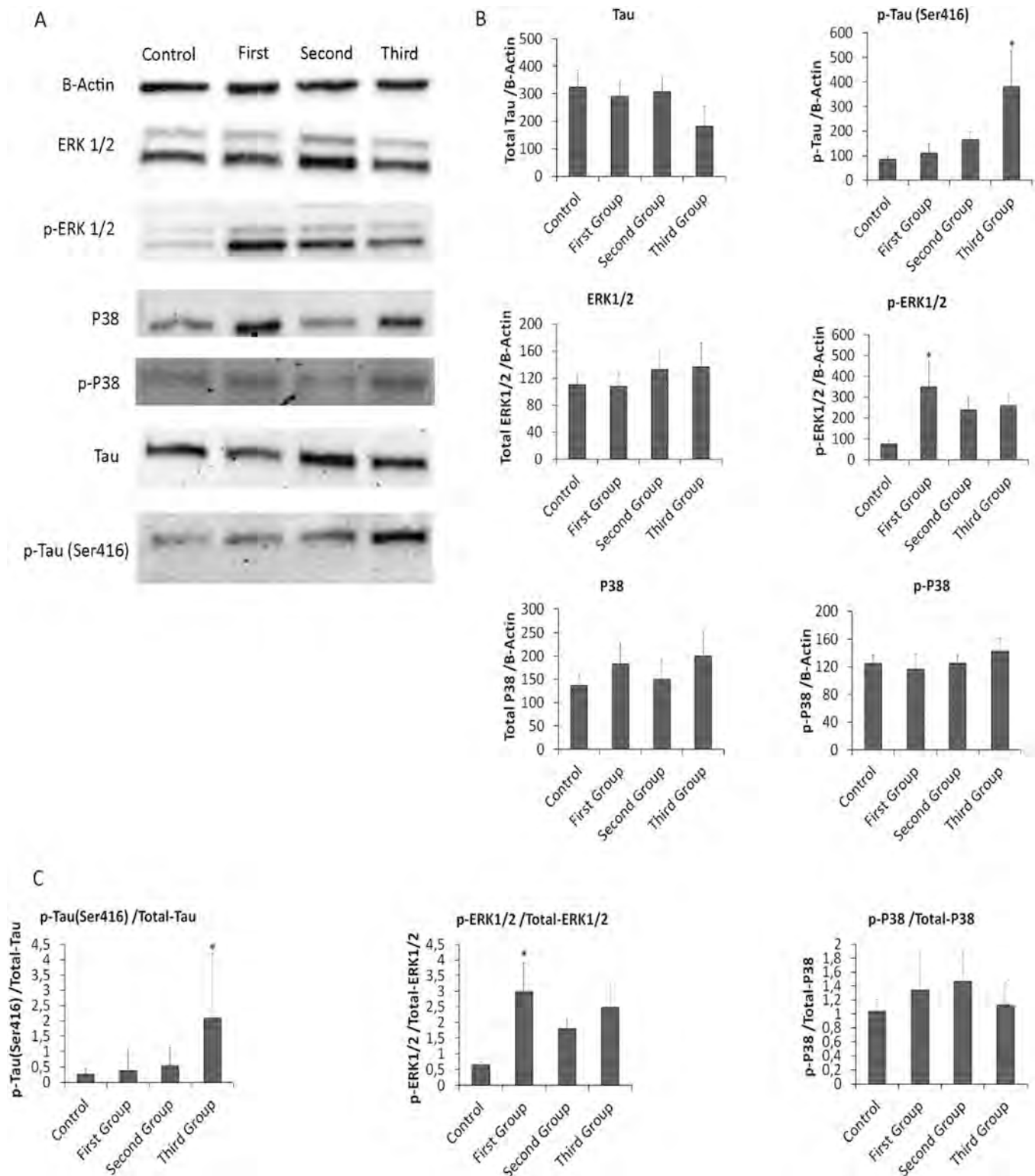


Fig. 7. Representative immunoblots of western blotting analysis in rats after exposure to pre and postnatal 2450 MHz CW-RFR for 12 h/day during experiment into second generations adult male rats (a) The phosphorylation and unphosphorylation status of Tau, ERK1/2 and p38 are presented (b) and the ratio of phosphoprotein over total protein (c), which was expressed according to data in (b) in second generations adult male rats as followed, the ratio of p-TauSer416 over total-Tau, ratio of p-ERK1/2 over total-ERK and ratio of p-P38 over total-P38. * depict denotes significant difference than control group (n = 6 per group). Statistical comparisons were evaluated by one-way ANOVA followed by LSD test. Statistical significance was accepted at $P < 0.05$.

process is completed before birth (Guidi et al., 2005) and neurons are very responsive to EMF (Aldinucci et al., 2009). However, the results of some studies reported that “EMFs can affect neuronal functions, including regulation of synaptic plasticity, neurotransmitter release,

neuronal survival, learning, and memory” (Sakatani et al., 2002; Manikonda et al., 2007).

Our most critical histopathological findings of fetuses were hemorrhagic areas and the irregular cellular localization in the cortex in

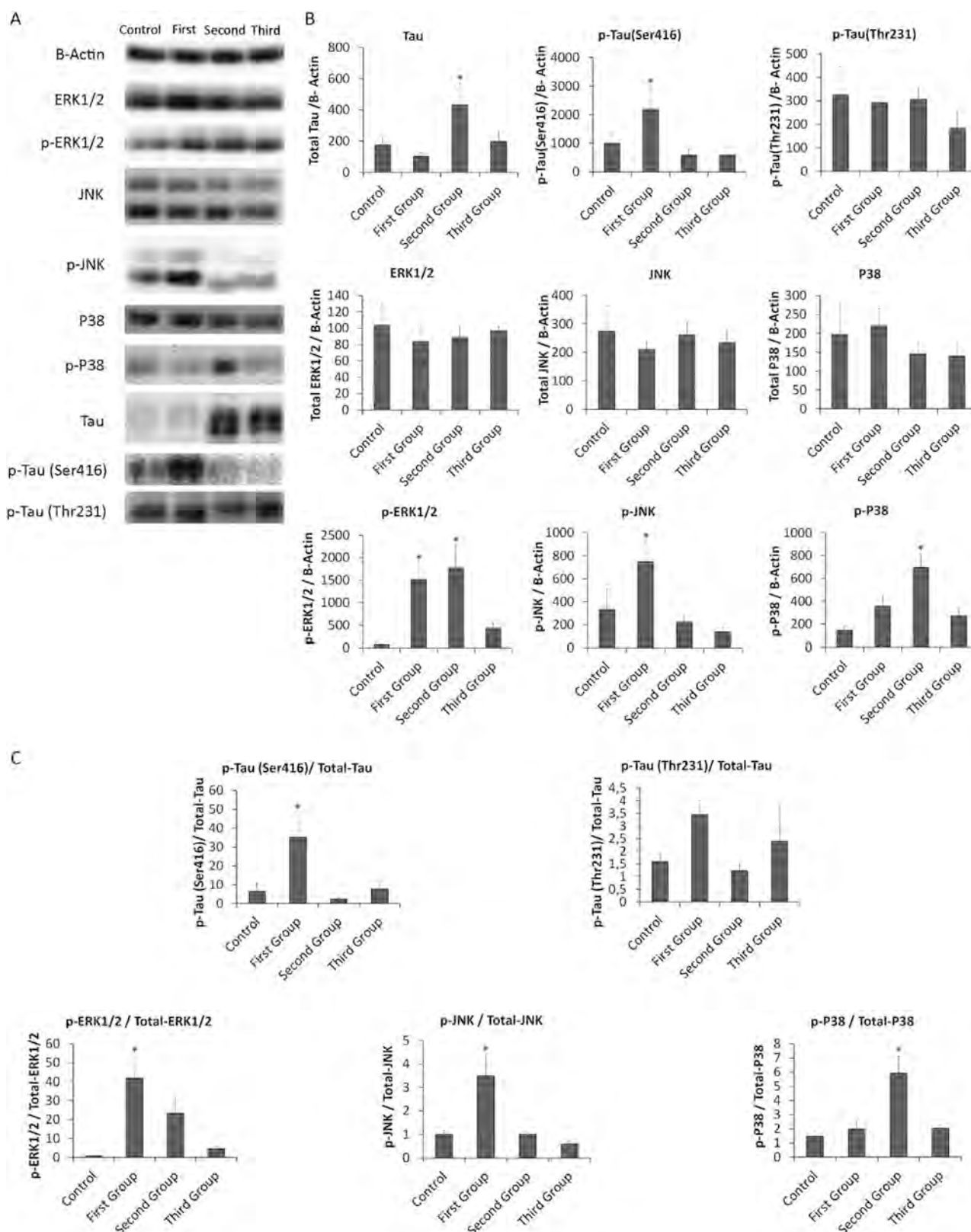


Fig. 8. Representative immunoblots of western blotting analysis in rats after exposure to pre and postnatal 2450 MHz CW-RFR for 12 h/day during experiment into third generations adult male rats (a) The phosphorylation and unphosphorylation status of Tau, ERK1/2, JNK and p38 are presented (b) and the ratio of phosphoprotein over total protein (c), which was expressed according to data in (b) in third generations adult male rats as followed, the ratio of p-TauSer416 over total-Tau, ratio of p-TauThr231 over total-Tau, ratio of p-ERK1/2 over total-ERK, ratio of p-JNK over total-JNK and ratio of p-P38 over total-P38. * depict denotes significant difference than control group (n = 6 per group). Statistical comparisons were evaluated by one-way ANOVA followed by LSD test. Statistical significance was accepted at $P < 0.05$.

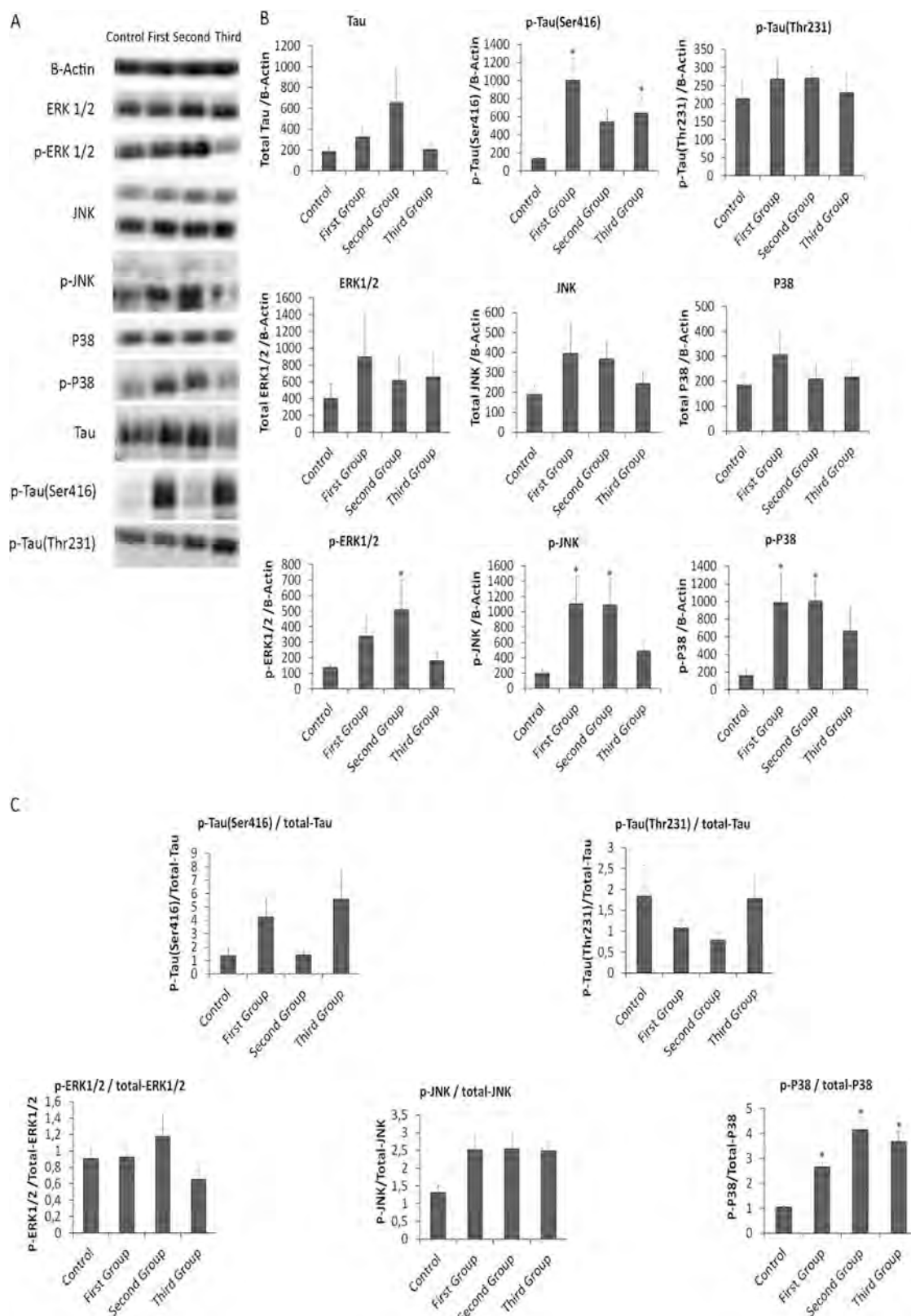


Fig. 9. Representative immunoblots of western blotting analysis in rats after exposure to pre and postnatal 2450 MHz CW-RFR for 12 h/day during experiment into fourth generations adult male rats (a) The phosphorylation and unphosphorylation status of Tau, ERK1/2, JNK and p38 are presented (b) and the ratio of phosphoprotein over total protein (c), which was expressed according to data in (b) in fourth generations adult male rats as followed, the ratio of p-TauSer416 over total-Tau, ratio of p-TauThr231 over total-Tau, ratio of p-ERK1/2 over total-ERK, ratio of p-JNK over total-JNK and ratio of p-P38 over total-P38. * depict denotes significant difference than control group (n = 6 per group). Statistical comparisons were evaluated by one-way ANOVA followed by LSD test. Statistical significance was accepted at $P < 0.05$.

exposed rats of the 1st and 2nd groups of the second-generation. Vascular structures were observed in exposed rats of the 1st, 2nd, and 3rd groups of the third-and fourth-generation. The vascular patterns of brain tumors play a crucial prognostic role in direct relation to tumor growth (Rong et al., 2006; Das and Marsden (2013); Rao et al., 2016; Tastekin et al., 2016). The effect of EMF on the central nervous system is of particular interest since disorders of the central nervous system are associated with many abnormalities in the functioning of the body and the possible carcinogenic effect of EMF. The main attention in evaluating the RF-EMF health effects has been directed to risk of tumors (Carlberg et al., 2013; Miller et al., 2019; Choi et al., 2020). Due to the long latent period, diverse exposure conditions, and biological parameters, the causal relationship is difficult to reveal. The recent meta-analyses of case-control studies found that cellular phone use with cumulative call time of more than 1000 h statistically significantly increased the risk of tumors (Choi et al., 2020).

Prenatal exposure results in lower birth weight, smaller size and significant impairment of the hippocampus, glial cells, and pyramidal cells (Dasdag et al., 2000; Bas et al., 2009; Hosseini-Sharifabad et al., 2012; Bas et al., 2013). Stasinopoulou et al. found that “pyramidal cell loss and glial fibrillary acidic protein (GFAP) over-expression were detected in the CA4 region of the hippocampus of the 22-day old to either during prenatal life or both pre-and postnatally” (Stasinopoulou et al., 2016). Balassa et al. demonstrated that “the basic excitability of hippocampal slices (measured as amplitude of population spikes) was increased by both types of treatment (fetal 0.5 mT, newborn 3 mT)” (Balassa et al. (2013)).

4.2. Adult female brain tissue

Salford et al. reported that “prenatal RFR exposure can cause cell death, inhibition of the differentiation of neural stem cells and alteration in the neurogenesis in both the developing and adult brain” (Salford et al., 2003). Ait-Aïssa et al. stated that “gestational and early life repeated exposure to WiFi (2 h/day; SAR = 0.08, 0.4, and 4 W/kg) has no deleterious effects on the brains in case of young rats” (Ait-Aïssa et al., 2013). Inversely, impaired neurite outgrowth on neuronal cells has been demonstrated in embryonic neural stem cells exposed to RFR (Chen et al. (2014)).

Our most critical histopathological findings in female rats were the hemorrhagic areas and the irregular cellular localization in the cortex, especially in the exposed rats of the 1st and 2nd groups of the second-generation compared to the sham control group. Histopathological findings were more minor in the 3rd group compared to the 1st and 2nd groups. Vascular dilatation areas and cellular localization irregularity were observed in some cortex regions especially in exposed rats of the 1st and 2nd groups of the third-and fourth-generation.

Similar to our study, some researchers have shown cellular losses and neuronal damage caused by exposure to EMF in many parts of the brain, including the cortex, basal ganglia, hippocampus, and cerebellum (Erdem et al., 2016; Kıvrak, 2014). A continuous 900 MHz exposure to EMF during a gestational period can cause developmental and histopathological changes in the human fetal spinal cord, such as vacuolization, atrophy, and thickening (İkinci et al., (2016)).

It is also revealed that “RFR exposure can cause shrinkage of pyramidal neurons, presence of mild perivascular and perineural edema, and some vacuolation of neurons and glial cells derived from mouse great hemispheres, reduction of Purkinje cells, vacuolisation of neurons and glial cells, and interstitial edema in the cerebellum” (Zymantiene et al., 2020). Similarly suggested that pyramidal neuron loss and histopathological changes in the cornu ammonium of 8-week-old male rats may be due to 900 MHz EMF exposure (Sahin et al., 2015).

4.3. Adult male brain tissue

Results of some studies indicated that “700 MHz RFR can alter

electrical activity in hippocampal slices of the rat brain (Tattersall et al., 2001) and 1800 MHz (15 min per day for eight days, SAR 2.40 W/kg) and has the potential to reduce the excitatory synaptic activity of cultured hippocampal neurons” (Xu et al., 2006).

As it is known, “Tau is an intracellular soluble protein that stabilizes microtubules in axons. The key features of neuropathological Alzheimer’s disease (AD) are extracellular senile plaques made up of aggregates of amyloid beta (A β) peptides, and intracellular deposits of hyperphosphorylated Tau protein” (Panza et al., 2019; Congdon and Sigurdsson (2018)). Previous reports suggested that “tau can be passively released into the extracellular space due to cell death or injury” (Hampel et al., 2010; Hesse et al., 2001). However, it is also reported that “tau is also actively secreted into the extracellular space in a regulated manner under physiological and pathological conditions” (Karch et al., 2012; Pooler et al., 2013; Yamada et al., 2011, 2014). On the other hand, some of the studies stated that “both total and phosphorylated tau (p-tau) are increased in the CSF of AD patients and predict the order and magnitude of pathologic processes that occur in AD patients” (Bateman et al., 2012; Fagan et al., 2014; Toledo et al., 2013).

Our study found changes in the function of the MAPK pathway, which affects cognitive processes such as learning and memory. Both pTau and pERK are increased in the exposed rats of the 1st and 3rd group of the second-generation compared to the other experimental groups.

pERK, pJNK and pTau were increased in the exposed rats of 1st group, and pERK, pP38 and total tau were increased in the exposed rats of the 2nd group of the third-generation compared to the other experimental groups. Also, pTau, pJNK and pP38 were increased in the exposed rats of the 1st group, and pERK, pJNK and pP38 were increased in the exposed rats of 2nd group of fourth generation compared to the other experimental groups. Also, pTau was increased in the exposed rats in the 3rd group of the fourth-generation compared to the other experimental groups.

Kim et al. observed that “neuronal cell bodies remained structurally stable but demyelination was induced in cortical neurons following prolonged RF-EMF” (Kim et al., 2017). Birks et al. reported that maternal cell phone use during pregnancy might be associated with an increased risk for offspring behavioral problems, particularly hyperactivity/inattention problems (Birks et al., 2017). Othman et al. showed that diverse types of prenatal Wi-Fi exposures provoked several behavioral and biochemical impairments at both juvenile and adult age of the offspring (Othman et al., 2017). Valbonesi et al. stated that “three different 1.8 GHz signals have not a potential to alter HSP70 (Heat shock protein 70) protein expression and three different MAPK signaling pathways” (Valbonesi et al., 2014).

In terms of knowing what pre-and post-natal RFR exposures cause over the four-generations, our study has the potential to contribute to the related field. However, when the studies discussed above are evaluated in general, we can say that a significant part of the studies supports our results. The results we obtained in our study showed that 2.4 MHz continuous RFRs could cause some changes in the brains of rats, and these may have the potential to prepare the ground for some diseases.

5. Conclusion

Results of the present study indicated that whole-body exposed to 2450 MHz CW-RFR increased the phosphorylation level of some MAPKs and Tau in the hippocampus in the brain of male rats. These findings suggest that exposure to RFR in rats may cause changes in the function of the MAPK pathway, which affects cognitive processes such as learning and memory, and may cause damage to both fetal and adult brain tissue. Furthermore, it causes histopathological changes in brain of fetus and female rat. Further studies are needed to elucidate the issue.

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Ethical statement

All tentative protocols applied on the rats were carried out with respect to the Principles of Laboratory Animal Care and the rules of Scientific and Ethics Committee of Experimental and Clinical Research Center (DEKAM) of Erciyes University. This research was confirmed by the Local Ethics Committee of Animal Experiments of Erciyes University (Decision No: 16/111).

CRediT authorship contribution statement

Burak Tan: Irradiation of animals, removal of tissues, monitoring and concluding stages of experiment processes. **Fazile Canturk Tan:** Intellectual infrastructure, methodology, funding acquisition, irradiation of animals, writing. **Betül Yalçın:** Removal of tissues, monitoring and concluding stages of experiment processes. **Suleyman Dasdag:** Intellectual infrastructure, writing, editing, supervision. **Arzu Hanım Yay:** Monitoring and concluding stages of experiment processes. **Korkut Yegin:** Editing, SAR measurement.

Conflict of interest

The authors declare their contributions to this manuscript and have no conflicts of interest.

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Effect of mobile phone usage duration during pregnancy on the general motor movements of infants

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ABSTRACT

Radiofrequency radiation (RFR) emitted from wireless devices increases rapidly and the most sensitive groups are pregnant women and children. Therefore, we aimed to evaluate the fidgety movements (FMs) and motor repertoires of the infants of pregnant women with different durations of mobile phone usage (DOMFU) in the prenatal period by performing a general movement assessment (GMA) using the Prechtl method. Infants suitable for the study were divided into 4 groups according to their mothers' duration of mobile phone usage during pregnancy, comprising those who did not talk on a mobile phone (Control Group, *n*: 31), those with mobile phone usage (MFU) of ~20 min a day (Group 1, *n*: 33), those with MFU of ~40 min a day (Group 2, *n*: 31), and those with MFU of ~2 h a day (Group 3, *n*: 28). The analysis showed that the abnormal fidgety (AF) and absent fidgety (F-), suboptimal motor optimality score (MOS) and reduced motor repertoire were statistically higher in Group 3 compared to the other groups. Normal posture and the quality of other movements were statistically higher in the Control, and Groups 1 and 2 compared to Group 3. According to the findings, infants of mothers with different DOMFU during pregnancy differed with regard to the quality of FMs, MOS, repertoire, posture and other movements. In conclusion, the findings suggested that there may be a relationship between prenatal RFR exposure and motor development in infants. More long-term studies are needed to determine whether these changes are temporary or permanent.

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Introduction

Along with developments in technology, the level of radiofrequency radiation (RFR) created by various wireless communication tools in the environment, especially mobile phones, is increasing rapidly, day-by-day, and has caused an environmental problem called Electromagnetic Pollution. Widespread use of mobile phones causes adverse effects on the nervous system, such as cognitive and neurological disorders [1,2], memory impairment [3], increased parasympathetic nerve activity [4], increased thyroid function [5], weakening of the immune system [6], increased permeability of the blood-brain barrier [7], changes in amygdala morphology and emotional behaviour [8], changes in cerebral cortex neurotransmitter release [9], cytotoxicity in hippocampal neuronal HT22 cells [9], and degenerative changes in hippocampus pyramidal cells [10], and has adverse effects on the nervous system.

Studies have shown that even gene and protein expression can be affected by radiofrequency radiation

(RFR) exposure [11–13]. Some authorities developed safety limits to protect the public against the negative health effects of RFR but the limits are presently valid for more susceptible groups, such as pregnant women, fetuses, or embryos [11].

Studies have suggested that prenatal RFR exposure might be associated with anomalies and growth retardation in fetuses or embryos [14,15], speech problems in children [16], oxidative stress in mothers and offspring [17], and changing electrophysiological properties in the Purkinje cerebellum neurons and ion currents [18]. It was reported that the research on the sensitivity of children to electromagnetic fields should be increased [11]. Research on the effects of RFR emitted in the environment by communication tools, such as mobile phones and Wi-Fi, on the foetus and mother is very limited. In most of the studies on the subject, the majority of which consist of animal studies, there are differences between the parameters, such as the experimental setups, techniques used and specific radiation absorption rates (SAR), etc [11].

The complex, fluid and variable-speed movements involving the whole body are called general movements (GMs). GMs begin at week 9 after menstruation and last up to 6 months postterm [19,20]. The quality of these GMs accurately reflects the infant's neural development and is an excellent way to identify early brain damage and dysfunction [20]. GMs differ in three periods: preterm, writhing, and fidgety. The normal movement patterns in newborns 3–5 months after birth are called fidgety movements (FMs) [21]. These FMs are evaluated as normal (F+), absent fidgety (F–), and abnormal fidgety (AF). AF and F– have high predictive values in predicting whether the baby is clinically developing normally or whether there is mild or severe neurological impairment [19]. In the last few decades, evidence has accumulated that FMs are a particularly accurate marker for the neurological outcome of high-risk infants. Recent systematic reviews and meta-analyses have recognized the qualitative analysis of GMs, using the Prechtl method, to be equally powerful or even more powerful than classical neurological examination and neuroimaging findings [22]. The GMs of 3–5-month-old infants include not only FMs, but also age-appropriate movement patterns and postural patterns [23].

In this study, we aimed to compare the FMs, motor optimality score (MOS), repertoire of co-occurring movements, posture and the quality of other movements in the 3–5 month period of the infants of women with mobile phone usage (MFU) of different durations during the prenatal period by performing a general movement assessment (GMA) using the Prechtl method.

Subjects and methods

Ethics statement

This study was initiated by the approval of Health Sciences University Van Training and Research Hospital Ethics Committee (Report No: 2021/20) after statistical power analysis. Parents or legal guardians provided written informed consent forms.

Subjects

A total of 123 infants, aged 3–5 months, who came to our polyclinic, were included in the study. The infants were divided into four groups according to the duration of their mothers' mobile phone usage during pregnancy: 1) those with no MFU (Control Group, *n*: 31), 2) those with MFU of ~20 min a day (Group 1, *n*: 33), 3) those with MFU of ~40 min a day (Group 2, *n*: 31), and 4) those with MFU of ~2 h a day (Group 3, *n*: 28). All of the parameters that may affect the infants' GMs and

neural development, such as birth weight, head circumference, birth length, gender, week of birth, status according to birth week, delivery type, and presence of foetal distress and meconium, were recorded. In addition, records were kept of some maternal information (maternal age, paternal consanguinity, hypertension, placental disease, systemic diseases, amniotic fluid status, vitamin, iron, vitamin D, folic acid use, toxoplasma, rubella, cytomegalovirus, herpes simplex, urinary tract infection, upper respiratory tract infection, vaginitis, chorioamnionitis, amount of medically exposed radiation, presence of base station, paternal smoking and alcohol habits, SAR values of mobile phones, and mobile phone usage times). The MOSs of the infants were calculated according to FMs and other accompanying movements.

Observational GMA

Observational GMA was performed by a paediatrician who had 7 years of GMA certification and experience. The evaluator completed the assessments blindly, without knowing the infants' clinical histories and or which group they belonged to. The Prechtl method was used for the observational GMA. The MOS of all of the infants were determined and the groups were compared with each other. The MOS, which has a maximum of 28 points and a minimum of 5 points, consists of five subsections and is scored as follows [24,25]:

1. Fidgety Movements: F+: 12 points, AF: 4 points, and F–: 1 point
2. Motor Repertoire: Age-appropriate motor repertoire: 4 points, decreased motor repertoire: 2 points, absence of age-appropriate motor repertoire: 1 point.
3. Motor Patterns (Except for FMs): Movement patterns can have a normal or abnormal appearance. Abnormal patterns are mostly circular arm movements and asymmetric segmental movements. It is 4 points if normal movement patterns are dominant, 2 points if normal and abnormal movements exist at an equal level, 1 point if abnormal movement patterns are dominant.
4. Posture: Normal postural pattern is 4 points, equal predominance of normal and abnormal postural pattern is 2 points, abnormal postural pattern is 1 point.
5. quality of Other Movements: 4 points are given if all movements are normally fluid, in various sequences and smooth. If the movements are jerky, rigid and less complex, they are considered abnormal, 2 points are given. If the

cramped-synchronized (CS) movement pattern is dominant, 1 point is given [23].

Statistical analyses

Whether the variables were suitable for normal distribution was tested using the Shapiro–Wilk test. Variables that did not fit the normal distribution were presented as median (minimum–maximum) values, the Mann–Whitney *U* test was used for comparisons between two independent groups, and the Kruskal–Wallis *H* test was used for comparisons of 3 or more independent groups. Categorical variables were expressed as frequencies and percentages, *n* (%), and the Fisher exact *chi*-square

test was used for the comparisons. Statistical analyses were conducted using IBM SPSS Statistics for Windows 22.0 (IBM Corp., Armonk, Ny, USA). Differences were considered statistically significant at the level of $p < 0.05$.

Results

The evaluations of the FMs, MOS, repertoire of co-occurring movements, posture, and quality of other movements of all infants are shown in Table 1. A minor neurological disorder (MND) was found in 1.6% of the infants.

During the prenatal period, the infants of mothers with different DOMFU differed in terms of the FMs, MOS, repertoire, posture, and quality of other movements ($p < 0.05$) (Table 2).

Table 1. Descriptive statistics of the FMs, MOS, repertoire of co-occurring movements, posture and quality of other movements.

	Frequency	Percentage
Fidgety		
F+	116	94.3
F–	2	1.6
AF	5	4.1
Total	123	100
MOS		
Optimal (25–28)	116	94.3
Suboptimal (24)	7	5.7
Total	123	100
Repertoire of co-occurring movements		
Age-adequate	119	96.7
Reduced	4	3.3
Total	123	100
Posture		
Normal	115	93.5
Normal = Abnormal	8	6.5
Total	123	100
Quality of other movements		
Normal > Abnormal	117	95.1
Normal = Abnormal	6	4.9
Total	123	100.0
Result		
Normal	121	98.4
MND	2	1.6
Total	123	100.0

Table 2. Investigation of the infants of mothers with different durations of mobile phone usage (DOMFU) during the prenatal period in terms of FMs, MOS, repertoire of co-occurring movements, posture and quality of other movements.

		Duration of use				<i>p</i> Value
		Control (<i>n</i> = 31)	20 min (<i>n</i> = 33)	40 min (<i>n</i> = 31)	2 h (<i>n</i> = 28)	
FMs	F+	31 (100)	33 (100)	29 (93.5)	23 (82.1)	0.011
	F–	0 (0)	0 (0)	0 (0)	2 (7.1)	
	AF	0 (0)	0 (0)	2 (6.5)	3 (10.7)	
MOS	Optimal (25–28)	31 (100)	33 (100)	30 (96.8)	22 (78.6)	<0.001
	Sub-optimal (24)	0 (0)	0 (0)	1 (3.2)	6 (21.4)	
Repertoire of co-occurring movements	Age-adequate	31 (100)	33 (100)	31 (100)	24 (85.7)	0.002
	Reduced	0 (0)	0 (0)	0 (0)	4 (14.3)	
	Absent	0 (0)	0 (0)	0 (0)	0 (0)	
Posture	Normal	31 (100)	33 (100)	29 (93.5)	22 (78.6)	0.001
	Normal = Abnormal	0 (0)	0 (0)	2 (6.5)	6 (21.4)	
	Abnormal	0 (0)	0 (0)	0 (0)	0 (0)	
Quality of other movements	Normal > Abnormal	31 (100)	33 (100)	31 (100)	22 (78.6)	<0.001
	Normal = Abnormal	0 (0)	0 (0)	0 (0)	6 (21.4)	
	Normal < Abnormal	0 (0)	0 (0)	0 (0)	0 (0)	

$p < 0.05$, Fisher's exact *chi*-square test.

Table 3. Investigation of the F- and AF status of the infants' FMs according to the MOS, repertoire, posture and quality of other movements and outcome.

	Fidgety		<i>p</i> Value
	Absent fidgety (<i>n</i> = 2)	Abnormal fidgety (<i>n</i> = 5)	
MOS			
Optimal (25–28)	0 (0)	1 (20)	0.999
Sub-optimal (24)	2 (100)	4 (80)	
Repertoire			
Age-adequate	0 (0)	3 (60)	0.429
Reduced	2 (100)	2 (40)	
Quality of other movements			
Normal > Abnormal	0 (0)	2 (40)	0.999
Normal = Abnormal	2 (100)	3 (60)	
Outcome			
Normal	0 (0)	5 (100)	0.048
MND	2 (100)	0 (0)	

Fisher's exact *chi*-square test

The AF and F- ($p < 0.05$), suboptimal MOSs ($p < 0.001$), reduced motor repertoire ($p < 0.05$) in Group 3 were statistically significantly higher than the other groups. Normal posture ($p = 0.001$) and quality of other movements ($p < 0.001$) were statistically significantly higher in the Control, and Groups 1 and 2 when compared to Group 3.

In the infants, the F- and AF did not differ in terms of the MOS, repertoire, or quality of other movements ($p > 0.05$). The F- and AF differed in terms of the outcome ($p < 0.05$). The infants with AF were neurologically normal, while the infants with F- developed MNDs (Table 3).

According to the findings, the repertoire of the infants differed according to the gestational week, and all of the infants with reduced repertoire were borderline premature. In other words, the rate of reduced repertoire was higher in the borderline premature infants when compared to the term, post-term and premature infants (Table 4).

In the results obtained, there was a relationship between the number of abortions by the mothers and the FMs. The number of F+ was higher in the infants of mothers who had not had an abortion when compared to those of mothers who had 4 or more abortions. The MOS and posture also differed according to the number of abortions. Optimal MOS and normal posture were observed more often in the infants of mothers who had not had an abortion when compared to those of mothers who had 4 or more abortions (Table 4).

There was a relationship between the MOS and posture and daily mobile phone usage frequency. The number of suboptimal MOS and normal = abnormal postures was higher in the infants of mothers with MFU > 4 times a day (Table 4). The other variables did not differ according to the quality of FMs, MOS, repertoire, posture or other movements.

Discussion

To the best of our knowledge, this was the first case-control study to examine FMs and motor repertoires in infants exposed to RFR emitted from mobile phones at different durations during the prenatal period. According to the findings, the infants of mothers with different DOMFU during the prenatal period differed with regard to the quality of the FMs, MOS, repertoire, posture, and other movements.

A baby's development is not only affected by individual factors related to biological and genetic characteristics. Environmental factors, such as microsystem (family, home, surroundings, peers, etc.), exosystem (extended family, neighbourhood, school, etc.) and macrosystem (community, economic system, culture, etc.) environments are also effective [23]. It is certain that people, and therefore pregnant women, will be exposed to these rays to a greater extent in today's world, where discussions that 5G technology can be more risky on living things and the environment continue to take place. This is because with the implementation of 5G technology, the cumulative RFR level in the environment will increase involuntarily and it will be inevitable for people to be exposed to these rays [26]. It does not seem plausible that RFR, whose levels in the environment are thought to increase tremendously, do not affect babies in the womb.

The idea that the human body can tolerate tens of times more radiation at millimeter wavelengths is based on a faulty modelling of the human body as an outer structure filled with a homogeneous fluid, along with the assumption that millimeter waves do not completely pass beyond the skin, completely ignore nerves, blood vessels and other electrically conductive structures that can carry radiation-induced currents deep into the body [11].

Table 4. Analysis of the confounding factors according to the FMs, MOS, repertoire, posture and quality of other movements.

	Fidgety movement			MOS		Repertoire of co-occurring movements			Posture		Quality of other movements	
	F+	F−	AF	Optimal (25–28)	Sub-optimal (24)	Age-adequate	Reduced	Normal	Normal = abnormal	Normal > abnormal	Normal = abnormal	
Daily mobile phone usage frequency												
Less than 2 times a day	8 (6.9)	0 (0)	0 (0)	8 (6.9)	0 (0)	8 (6.7)	0 (0)	8 (7)	0 (0)	6 (6.1)	0 (0)	
2–4 times a day	31 (26.7)	0 (0)	0 (0)	31 (26.7)	0 (0)	31 (26.1)	0 (0)	31 (27)	0 (0)	25 (25.5)	0 (0)	
More than 2 times a day	45 (38.8)	2 (100)	5 (100)	45 (38.8)	7 (100)	48 (40.3)	4 (100)	44 (38.3)	8 (100)	39 (39.8)	6 (100)	
Absent	32 (27.6)	0 (0)	0 (0)	32 (27.6)	0 (0)	32 (26.9)	0 (0)	32 (27.8)	0 (0)	28 (28.6)	0 (0)	
<i>p</i>	0.184			0.025		0.269		0.013		0.078		
Abortion												
Absent	95 (81.9)	2 (100)	2 (40)	95 (81.9)	4 (57.1)	96 (80.7)	3 (75)	94 (81.7)	5 (62.5)	95 (96.9)	4 (66.7)	
1	12 (10.3)	0 (0)	0 (0)	12 (10.3)	0 (0)	12 (10.1)	0 (0)	12 (10.4)	0 (0)	0 (0)	0 (0)	
2	3 (2.6)	0 (0)	1 (20)	3 (2.6)	1 (14.3)	4 (3.4)	0 (0)	3 (2.6)	1 (12.5)	0 (0)	0 (0)	
3	3 (2.6)	0 (0)	0 (0)	3 (2.6)	0 (0)	3 (2.5)	0 (0)	3 (2.6)	0 (0)	0 (0)	0 (0)	
4 or more	3 (2.6)	0 (0)	2 (40)	3 (2.6)	2 (28.6)	4 (3.4)	1 (25)	3 (2.6)	2 (25)	3 (3.1)	2 (33.3)	
<i>p</i>	0.041			0.029		0.378		0.041		0.259		
Gestational age (weeks)												
Term (37–42)	85 (73.3)	0 (0)	2 (40)	85 (73.3)	2 (28.6)	87 (73.1)	0 (0)	84 (73)	3 (37.5)	72 (73.5)	1 (16.7)	
Post-term (42+)	3 (2.6)	0 (0)	0 (0)	3 (2.6)	0 (0)	3 (2.5)	0 (0)	3 (2.6)	0 (0)	2 (2)	0 (0)	
Premature (29–32)	1 (0.9)	0 (0)	0 (0)	1 (0.9)	0 (0)	1 (0.8)	0 (0)	1 (0.9)	0 (0)	1 (1)	0 (0)	
Borderline premature (33–37)	27 (23.3)	2 (100)	3 (60)	27 (23.3)	5 (71.4)	28 (23.5)	4 (100)	27 (23.5)	5 (62.5)	23 (23.5)	5 (83.3)	
<i>p</i>	0.086			0.051		0.011		0.130		0.163		
Kinship												
Absent	54 (46.6)	2 (100)	3 (60)	54 (46.6)	5 (71.4)	56 (47.1)	3 (75)	53 (46.1)	6 (75)	55 (47)	4 (66.7)	
1st degree	44 (37.9)	0 (0)	2 (40)	44 (37.9)	2 (28.6)	45 (37.8)	1 (25)	44 (38.3)	2 (25)	44 (37.6)	2 (33.3)	
2nd degree	11 (9.5)	0 (0)	0 (0)	11 (9.5)	0 (0)	11 (9.2)	0 (0)	11 (9.6)	0 (0)	11 (9.4)	0 (0)	
Distant relative	5 (4.3)	0 (0)	0 (0)	5 (4.3)	0 (0)	5 (4.2)	0 (0)	5 (4.3)	0 (0)	5 (4.3)	0 (0)	
No Kinship Same Village	2 (1.7)	0 (0)	0 (0)	2 (1.7)	0 (0)	2 (1.7)	0 (0)	2 (1.7)	0 (0)	2 (1.7)	0 (0)	
<i>p</i>	0.902			0.752		0.805		0.668		0.884		
Hypertension												
Preeclampsia	23 (22.3)	0 (0)	2 (40)	23 (22.3)	2 (28.6)	25 (23.6)	0 (0)	23 (22.5)	2 (25)	19 (21.3)	1 (16.7)	
Eclampsia	11 (10.7)	0 (0)	1 (20)	11 (10.7)	1 (4.3)	11 (10.4)	1 (25)	11 (10.8)	1 (12.5)	10 (11.2)	1 (16.7)	
Not evaluated	6 (5.8)	0 (0)	0 (0)	6 (5.8)	0 (0)	6 (5.7)	0 (0)	6 (5.9)	0 (0)	6 (6.7)	0 (0)	
Absent	63 (61.2)	2 (100)	2 (40)	63 (61.2)	4 (57.1)	64 (60.4)	3 (75)	62 (60.8)	5 (62.5)	54 (60.7)	4 (66.7)	
<i>p</i>	0.695			0.818		0.430		0.999		0.999		
Birth												
Normal spontaneous vaginal delivery	55 (47.4)	1 (50)	2 (40)	55 (47.4)	3 (42.9)	57 (47.9)	1 (25)	54 (47)	4 (50)	45 (45.9)	3 (50)	
Emergency caesarean spinal anaesthesia	5 (4.3)	1 (50)	0 (0)	5 (4.3)	1 (14.3)	5 (4.2)	1 (25)	5 (4.3)	1 (12.5)	4 (4.1)	1 (16.7)	
Emergency caesarean general anaesthesia	10 (8.6)	0 (0)	0 (0)	10 (8.6)	0 (0)	10 (8.4)	0 (0)	10 (8.7)	0 (0)	8 (8.2)	0 (0)	
Elective caesarean spinal anaesthesia	27 (23.3)	0 (0)	1 (20)	27 (23.3)	1 (14.3)	28 (23.5)	0 (0)	27 (23.5)	1 (12.5)	24 (24.5)	0 (0)	
Elective caesarean general anaesthesia	19 (16.4)	0 (0)	2 (40)	19 (16.4)	2 (28.6)	19 (16)	2 (50)	19 (16.5)	2 (25)	17 (17.3)	2 (33.3)	
<i>p</i>	0.405			0.511		0.098		0.594		0.269		
Foetal distress												
Present	22 (19)	1 (50)	0 (0)	22 (19)	1 (14.3)	22 (18.5)	1 (25)	22 (19.1)	1 (12.5)	18 (18.4)	1 (16.7)	
Absent	91 (78.4)	1 (50)	5 (100)	91 (78.4)	6 (85.7)	94 (79)	3 (75)	90 (78.3)	7 (87.5)	77 (78.6)	5 (83.3)	

(Continued)

Table 4. (Continued)

Not evaluated	3 (2.6)	0 (0)	0 (0)	3 (2.6)	0 (0)	3 (2.6)	0 (0)	3 (3.1)	0 (0)
<i>p</i>	0.372			0.999		0.618		0.999	
Meconium									
Present	14 (12.1)	1 (5.0)	0 (0)	14 (12.1)	1 (14.3)	14 (11.8)	1 (12.5)	14 (14.3)	1 (16.7)
Absent	102 (87.9)	1 (5.0)	5 (100)	102 (87.9)	6 (85.7)	105 (88.2)	7 (87.5)	84 (85.7)	5 (83.3)
<i>p</i>	0.319			0.999		0.410		0.999	
Toxoplasma, rubella, cytomegalovirus, herpes simplex. HIV									
Absent	114 (98.3)	2 (100)	5 (100)	114 (98.3)	7 (100)	117 (98.3)	8 (100)	96 (98)	6 (100)
Not evaluated	2 (1.7)	0 (0)	0 (0)	2 (1.7)	0 (0)	2 (1.7)	0 (0)	2 (2)	0 (0)
<i>p</i>	0.999			0.999		0.999		0.999	
Placental disease									
Absent	109 (94)	2 (100)	4 (80)	109 (94)	6 (85.7)	111 (93.3)	7 (87.5)	92 (93.9)	6 (100)
Placenta previa	1 (0.9)	0 (0)	0 (0)	1 (0.9)	0 (0)	1 (0.8)	0 (0)	1 (1)	0 (0)
Placental Insufficiency	6 (5.2)	0 (0)	1 (20)	6 (5.2)	1 (14.3)	7 (5.9)	1 (12.5)	5 (5.1)	0 (0)
<i>p</i>	0.383			0.383		0.999		0.999	
Systemic disease									
Diabetes mellitus	1 (0.9)	0 (0)	0 (0)	1 (0.9)	0 (0)	1 (0.8)	0 (0)	0 (0)	0 (0)
Hyperthyroidism	1 (0.9)	0 (0)	0 (0)	1 (0.9)	0 (0)	1 (0.8)	0 (0)	1 (1)	0 (0)
Vasculitis	3 (2.6)	0 (0)	0 (0)	3 (2.6)	0 (0)	3 (2.5)	0 (0)	2 (2)	0 (0)
Absent	111 (95.7)	2 (100)	5 (100)	111 (95.7)	7 (100)	114 (95.8)	8 (100)	95 (96.9)	6 (100)
<i>p</i>	0.999			0.999		0.999		0.999	
Amniotic fluid									
Polyhydramnios	4 (3.4)	0 (0)	0 (0)	4 (3.4)	0 (0)	4 (3.4)	0 (0)	4 (4.1)	0 (0)
Oligohydramnios	4 (3.4)	0 (0)	1 (20)	4 (3.4)	1 (14.3)	4 (3.4)	1 (12.5)	3 (3.1)	1 (16.7)
Normal	108 (93.1)	2 (100)	4 (80)	108 (93.1)	6 (85.7)	111 (93.3)	7 (87.5)	91 (92.9)	5 (83.3)
<i>p</i>	0.421			0.421		0.265		0.389	
Vitamin									
Not used	1 (0.9)	0 (0)	0 (0)	1 (0.9)	0 (0)	1 (0.8)	0 (0)	1 (1)	0 (0)
Irregular usage	56 (48.3)	2 (100)	2 (40)	56 (48.3)	4 (57.1)	58 (48.7)	5 (62.5)	47 (48)	3 (50)
Regular usage	59 (50.9)	0 (0)	3 (60)	59 (50.9)	3 (42.9)	60 (50.4)	3 (37.5)	50 (51)	3 (50)
<i>p</i>	0.468			0.731		0.999		0.999	
Iron									
Not used	10 (8.6)	0 (0)	0 (0)	10 (8.6)	0 (0)	10 (8.4)	0 (0)	7 (7.1)	0 (0)
Irregular usage	42 (36.2)	2 (100)	2 (40)	42 (36.2)	4 (57.1)	44 (37)	5 (62.5)	36 (36.7)	3 (50)
Regular usage	64 (55.2)	0 (0)	3 (60)	64 (55.2)	3 (42.9)	65 (54.6)	3 (37.5)	55 (56.1)	3 (50)
<i>p</i>	0.498			0.584		0.999		0.793	
Vitamin D									
Irregular usage	53 (45.7)	2 (100)	2 (40)	53 (45.7)	4 (57.1)	55 (46.2)	5 (62.5)	44 (44.9)	3 (50)
Regular usage	63 (54.3)	0 (0)	3 (60)	63 (54.3)	3 (42.9)	64 (53.8)	3 (37.5)	54 (55.1)	3 (50)
<i>p</i>	0.409			0.703		0.999		0.999	
Folic acid									
Irregular usage	53 (45.7)	2 (100)	2 (40)	53 (45.7)	4 (57.1)	55 (46.2)	5 (62.5)	44 (44.9)	3 (50)
Regular usage	63 (54.3)	0 (0)	3 (60)	63 (54.3)	3 (42.9)	64 (53.8)	3 (37.5)	54 (55.1)	3 (50)
<i>p</i>	0.409			0.703		0.999		0.999	
Radiation									
Absent	110 (94.8)	2 (100)	5 (100)	110 (94.8)	7 (100)	113 (95)	8 (100)	93 (94.9)	6 (100)
Unknown	6 (5.2)	0 (0)	0 (0)	6 (5.2)	0 (0)	6 (5)	0 (0)	5 (5.1)	0 (0)
<i>p</i>	0.999			0.999		0.999		0.999	
Urinary tract infections									
Present	62 (53.4)	1 (50)	1 (20)	62 (53.4)	2 (28.6)	62 (52.1)	2 (25)	51 (52)	2 (33.3)

(Continued)

Table 4. (Continued)

Absent	54 (46.6)	1 (50)	4 (80)	54 (46.6)	5 (71.4)	57 (47.9)	2 (50)	53 (46.1)	6 (75)	47 (48)	4 (66.7)
<i>p</i>		0.347			0.259	0.999			0.151		0.432
Upper respiratory infections											
Present	45 (38.8)	1 (50)	1 (20)	45 (38.8)	2 (28.6)	45 (37.8)	2 (50)	45 (39.1)	2 (25)	36 (36.7)	2 (33.3)
Absent	71 (61.2)	1 (50)	4 (80)	71 (61.2)	5 (71.4)	74 (62.2)	2 (50)	70 (60.9)	6 (75)	62 (63.3)	4 (66.7)
<i>p</i>		0.833			0.707	0.636			0.709		0.999
Vaginitis											
Present	52 (44.8)	1 (50)	1 (20)	52 (44.8)	2 (28.6)	52 (43.7)	2 (50)	52 (45.2)	2 (25)	44 (44.9)	2 (33.3)
Absent	64 (55.2)	1 (50)	4 (80)	64 (55.2)	5 (71.4)	67 (56.3)	2 (50)	63 (54.8)	6 (75)	54 (55.1)	4 (66.7)
<i>p</i>		0.694			0.465	0.999			0.464		0.691
Placental disease											
Present	29 (25)	1 (50)	1 (20)	29 (25)	2 (28.6)	29 (24.4)	2 (50)	29 (25.2)	2 (25)	25 (25.5)	2 (33.3)
Absent	87 (75)	1 (50)	4 (80)	87 (75)	5 (71.4)	90 (75.6)	2 (50)	86 (74.8)	6 (75)	73 (74.5)	4 (66.7)
<i>p</i>		0.778			0.999	0.263			0.999		0.648
Smoking habit											
Not smoking	79 (68.1)	0 (0)	3 (60)	79 (68.1)	3 (42.9)	81 (68.1)	1 (25)	79 (68.7)	3 (37.5)	64 (65.3)	2 (33.3)
Smoking	37 (31.9)	2 (100)	2 (40)	37 (31.9)	4 (57.1)	38 (31.9)	3 (75)	36 (31.3)	5 (62.5)	34 (34.7)	4 (66.7)
<i>p</i>		0.110			0.220	0.107			0.115		0.188
Alcohol consumption during pregnancy											
Present	1 (0.9)	0 (0)	0 (0)	1 (0.9)	0 (0)	1 (0.8)	0 (0)	1 (0.9)	0 (0)	1 (1)	0 (0)
Absent	115 (99.1)	2 (100)	5 (100)	115 (99.1)	7 (100)	118 (99.2)	4 (100)	114 (99.1)	8 (100)	97 (99)	6 (100)
<i>p</i>		0.813			0.999	0.999			0.999		0.999
Number of cigarettes											
0	79 (68.1)	0 (0)	3 (60)	79 (68.1)	3 (42.9)	81 (68.1)	1 (25)	79 (68.7)	3 (37.5)	64 (65.3)	2 (33.3)
1–3 cigarettes	19 (16.4)	2 (100)	0 (0)	18 (15.5)	3 (42.9)	19 (16)	2 (50)	18 (15.7)	3 (37.5)	17 (17.3)	3 (50)
4–10 cigarettes	17 (14.7)	0 (0)	2 (40)	18 (15.5)	1 (14.3)	18 (15.1)	1 (25)	17 (14.8)	2 (25)	16 (16.3)	1 (16.7)
More than 10	1 (0.9)	0 (0)	0 (0)	1 (0.9)	0 (0)	1 (0.8)	0 (0)	1 (0.9)	0 (0)	1 (1)	0 (0)
<i>p</i>		0.077			0.185	0.132			0.152		0.156
Wi-Fi at home											
Not available	55 (47.4)	0 (0)	3 (60)	56 (48.3)	2 (28.6)	57 (47.9)	1 (25)	55 (47.8)	3 (37.5)	50 (51)	1 (16.7)
Available	39 (33.6)	1 (50)	2 (40)	38 (32.8)	4 (57.1)	40 (33.6)	2 (50)	38 (33)	4 (50)	30 (30.6)	4 (66.7)
Available in the building	22 (19)	1 (50)	0 (0)	22 (19)	1 (14.3)	22 (18.5)	1 (25)	22 (19.1)	1 (12.5)	18 (18.4)	1 (16.7)
<i>p</i>		0.470			0.410	0.525			0.626		0.191
Wi-Fi in workplace											
Not available	98 (85.2)	0 (0)	4 (80)	98 (85.2)	0 (0)	100 (84.7)	2 (50)	97 (85.1)	5 (62.5)	98 (84.5)	4 (66.7)
Available	17 (14.8)	2 (100)	1 (20)	17 (14.8)	2 (100)	18 (15.3)	2 (50)	17 (14.9)	3 (37.5)	18 (15.5)	2 (33.3)
<i>p</i>		0.064			0.086	0.125			0.123		0.255
Base Station Nearby											
Available	27 (23.3)	0 (0)	0 (0)	27 (23.3)	0 (0)	27 (22.7)	0 (0)	27 (23.5)	0 (0)	27 (23.1)	0 (0)
Not available	89 (76.7)	2 (100)	5 (100)	89 (76.7)	7 (100)	92 (77.3)	4 (100)	88 (76.5)	8 (100)	90 (76.9)	6 (100)
<i>p</i>		0.748			0.346	0.575			0.198		0.337

p < 0.0; Fisher's exact chi-square test.

The offspring of mice exposed to RFR in the prenatal period showed decreased memory, hyperactivity and glutamatergic synaptic transmission disorder in the pyramidal cells in the prefrontal cortex [27]. Another study detected changes in astrocytic and apoptotic responses in different brain regions of rats exposed to RFR during the prenatal period (2h/day, 5 days/week, 2 weeks) and in the first weeks of the postnatal period. However, RFR did not create a permanent activation of astroglia in the brains of rats and did not trigger apoptosis [28]. There was a significant reduction in the number of dentate gyrus granule cells in the hippocampus [29], and the number of pyramidal cells [30] and the presence of picnotic cells in the cornu ammonis region of the hippocampus in rats exposed to RFR (60 min/day, 900 MHz) during the prenatal period [31]. Moreover, it was stated that exposure to RFR (900 MHz, pulsed) during pregnancy can change the electrophysiological properties of the Purkinje neurons of rats in the postnatal period, but these changes are not sufficient to affect cerebellum-related functional functions [18]. On the other hand exposure to RFR in the prenatal period did not cause any changes in the expression of the *c-fos* gene, which is an indicator of neural stress in mouse brains [32], and did not cause any measurable cognitive deficit [33].

In a study conducted by Vrijheid et al. [34] on 530 children, there were very small differences between the children of mothers who had used mobile phones during the prenatal period and those who had not. The children of mothers who had used mobile phones had higher mental scores but lower psychomotor development than those whose mothers had not used mobile phones. Kane [35] also published a hypothesis suggesting that the increase in the incidence of autism in children in recent years may be related to the dramatically increasing use of mobile phones with advancing technology. Moreover, researchers have found an association between exposure to RFR emitted from mobile phones, both prenatally and postnatally, and migraine and other headache disorders [36], and between exposure to RFR emitted from mobile phones during pregnancy and behavioural disorders, such as hyperactivity and emotional problems at school [37]. The average cognitive scores and mental activation levels were lower in children with high maternal cell phone usage intensity [38]. RFR exposure with environmental factors at different frequencies and intensities has been reported to induce neurobiological disorders [39]. However, another study conducted on 6 and 18-month-old babies, found no relationship between prenatal cell phone use and

delays in motor and cognitive/language development [40]. In another study, while there was no relationship between mobile phone usage during pregnancy and foetal development or birth weight, it was found that it could cause preterm birth [41].

In the present study, the AF and F- ($p < 0.05$), sub-optimal MOS scores ($p < 0.001$), reduced motor repertoire ($p < 0.05$) values were statistically higher in Group 3 when compared to the other groups ($p < 0.05$). Normal posture ($p = 0.001$) and the quality of other movements were statistically higher in the Control, and Groups 1 and 2 when compared to Group 3 ($p < 0.001$).

Abnormal GMs and postural anomalies occurring at 3–5 months of age may indicate cognitive anomalies that will emerge in the following years [21]. The infants of mothers with MFU of ~2 h a day had a lower MOS values when compared to the children of mothers with MFU of other durations. These findings were similar to those found in infants at risk for motor problems [42,43]. Similarly, the MOS was significantly lower in infants of mothers who used drugs/alcohol [24]. Studies in literature showed that the GMA, including MOS, can provide important information about the later neurodevelopmental functions of infants [25].

According to the results obtained, there was no correlation between the F- and AF levels of the infants and the MOS, repertoire, and the quality of other movements. However, they differed with regard to the outcome. It was determined that the infants with AF were neurologically normal, while the infants with F- had minor neurological impairment. In the literature, abnormal simultaneous motor repertoire was associated with later impaired cognitive and motor outcomes, even in high-risk infants with FMs [44].

FM development in GMs, which occurs in infants at post-term 3–5 months, is synchronized with a series of motor repertoires, postural patterns and other age-appropriate movements [22]. An evaluation of all these movements together provides important information regarding the infant's subsequent motor function [42]. This period is ideal for evaluating neurobehavioral repertoires and predicting outcomes in high-risk infants [22]. Some of the studies applying a similar approach found that a low MOS score was associated with motor and language dysfunction, minor neurological dysfunctions, or school-age learning difficulties in the toddler age [42].

In this study, we analyzed statistically the paternal and environmental variables that may affect the infant's motor development, such as maternal and

paternal age, maternal and paternal education and occupation, weight gained during pregnancy, infant's weight according to gestational week, infant's head circumference and height, mother's history of pregnancy, number of abortions and stillbirths, cell phone SAR value, daily cell phone usage frequency, gestational week, maternal diseases (hypertension, systemic disease, vaginitis, placental infection, urinary tract infection, upper respiratory tract infection), mode of delivery, foetal distress and meconium, vitamin and folic acid use during pregnancy, alcohol and cigarette use, at home and at work Wi-Fi usage, presence of base stations around, radiation exposure during pregnancy, and the infants' FM, MOS, repertoire, posture and the quality of other movements. In addition, we also evaluated whether the pregnant women included in the study had complaints of dizziness, restlessness, ear pain, facial sensitivity or burning. The data obtained revealed that the FMs differed according to the number of abortions. The normal FM level observed in the infants of the pregnant women who had not had an abortion was higher than in those of the pregnant women who had 4 or more abortions. A difference was also observed between the number of abortions and the MOS and posture. The optimal MOS and normal posture observed in the infants of pregnant women who had not had an abortion were higher than those whose mothers had 4 or more abortions. Moreover, it was observed that there may be a relationship between the MOS, posture and daily cell phone usage frequency. For example, the sub-optimal MOS and normal=abnormal postures were higher in those who used cell phones for more than 4 times a day. However, it was understood that the other variables did not differ according to the nature of the FM, MOS, repertoire, posture and other movements. The findings showed that all of the infants with reduced repertoire were borderline premature. In other words, the incidence of reduced repertoire in borderline premature infants was higher than in the term, post-term and premature infants.

In addition to a causal relationship between the minor changes observed in the present study and prenatal mobile phone usage, other confounding parameters should also be considered. Therefore, it is difficult to predict for now what kind of problems prenatal exposure to RFR emitted from mobile phones would cause in these children in the future. However, it seems inevitable that RFR levels, which will reach incredible levels in the future, will somehow affect babies in the womb. Therefore, such studies should be long term and the children included in the study

should be followed up at intervals of 5 or 10 years. Moreover, whether the changes obtained in this study are reversible or irreversible is the subject of another study. Therefore, much work is needed to elucidate this issue. The data herein suggested that there may be a relationship between prenatal RFR exposure, and the parameters discussed herein. The topicality and interestingness of the subject should be kept on the agenda.

Conclusions

The data obtained in this study suggest that there may be a relationship between prenatal RFR exposure and infant motor development. More long-term studies are needed to determine whether these changes are temporary or permanent.

Data availability statement

The data supporting the findings of this study are available within the article.

Disclosure statement

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ORIGINAL ARTICLE



Role of 2.4 GHz radiofrequency radiation emitted from Wi-Fi on some miRNA and fatty acids composition in brain

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ABSTRACT

The purpose of this study is to investigate the effects of 2.4 GHz Wi-Fi exposure, which is continuously used in the internet connection by mobile phones, computers and other wireless equipment, on microRNA and membrane and depot fatty acid composition of brain cells. Sixteen Wistar Albino rats were divided equally into two groups such as sham and exposure. The rats in the experimental group ($n = 8$) were exposed to 2.4 GHz RFR emitted from a Wi-Fi generator for 24 h/day for one year. The animals in the control group ($n = 8$) were kept under the same conditions as the experimental group, but the Wi-Fi generator was turned off. At the end of the study, rats were sacrificed and brains were removed to analyze miRNA expression and membrane and depot fatty acids of brain cells. We analyzed the situation of ten different miRNA expressions and nineteen fatty acid patterns in this study. We observed that long-term and excessive exposure of 2.4 GHz Wi-Fi radiation increased rno-miR-181a-5p, phosphatidylserine (PS) and triacylglycerol (TAG) in the brain. In conclusion, 2.4 GHz Wi-Fi exposure has the potential to alter rno-miR-181a-5p expression and the fatty acid percentage of some membrane lipids such as phospholipid (PL), phosphatidylserine (PS) and triacylglycerol (TAG), which are depot fats in the brain. However, the uncontrolled use of RFRs, whose use and diversity have reached incredible levels with each passing day and which are increasing in the future, may be paving the way for many diseases that we cannot connect with today.

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wireless communication;
miRNAs; fatty acid
composition of membrane
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Introduction

As it is known, the International Agency for Research on Cancer in 2011 classified radiofrequency radiations (RFRs) as possible carcinogen (2B) to humans, opening this field for further investigation (International Agency for Research on Cancer (IARC) 2011). However, we have very limited information on the relation between microRNAs (miRNAs, miRs), fatty acids in brain and RFRs, which is very important to understand the effects of electromagnetic pollution emitted by all wireless equipment that is uncontrolled and widely used in our daily life. In our previous works we indicated that long term and excessive use of 900 MHz and 2.4 GHz RFR radiofrequency radiation altered microRNA expression in brain (Dasdag et al., 2015a; 2015b). We investigated the effects of 900 MHz and 2.4 GHz RFR on some miRNAs namely, rno-miR-9-5p, rno-miR-29a-3p, rno-

miR-106b-5p, rno-miR-107 and rno-miR-125a-3p in brain. In the 900 MHz exposure study, rats were exposed to 900 MHz RF radiation for 3 h per day (7 days a week) for 12 months (one year) in the exposure group and we found that 900 MHz RFR exposure only decreased rno-miR107 expression (Dasdag et al., 2015a). In the 2.4 GHz Wi-Fi exposure study, rats were exposed to 2.4 GHz RFRs emitted from Wi Fi generator for 24 hours a day for 12 months (one year) in the exposure group and we found that one-year exposure of 2.4 GHz Wi-Fi radiation altered expression of some miRNAs such as rno-miR-106b-5p and rno-miR-107. The results of this study indicated that rno-miR-107 expression was 3.3 times and rno-miR-106b-5p expression was 3.65 times lower in the exposure group than the control group (Dasdag et al., 2015b). We also investigated the effects

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of 900 MHz RFR with same exposure setup on ten miRNAs in 2019 and found that chronic exposure of RFR under investigation has potential to increase expression of rno-miR-145-5p (Dasdag et al., 2019).

Kumar R et al. (2021) exposed rats to 900 (5.84×10^{-4} W/kg), 1800 (5.94×10^{-4} W/kg) and 2.4 GHz (6.4×10^{-4} W/kg) RFRs for 2h/d for one, three and six-months and stated that “900–2450 MHz exposure led to hypermethylation of histone (H3K9) protein by upregulating euchromatic histone methyltransferase1 enzyme, whereas hypomethylation of DNA (5-Mc) by down-regulating DNA methyltransferase1 enzyme with increasing microwave frequency as well as exposure duration of Wistar rat” (Kumar et al., 2021).

Another important parameter in terms of electromagnetic pollution is the relation between RFR exposure and membrane fatty acids, which we have also very limited knowledge in this field

It is stated that “pain sensation can be changed upon the effect of extremely weak chemical and physical signals, having intensity even less than thermal threshold and non-linear dose-dependent character. Therefore, such a cellular target must have a quantum-mechanical nature” Ayrapetyan S (2018). However, Dutta et al indicated that “RFR affects both calcium-ion release and acetylcholinesterase activity in nervous system-derived cells in culture in a common dose-dependent manner” Dutta et al (1992). As it is also reported, “primary target for non-thermal biological effects of weak intensity radiation, is skin water contents the dissociation molecules of which have quantum sensitive characters. The product of water molecules dissociation which forming ROS serve one of important messengers through which circulating blood modulate the metabolism of different tissues. Since by fundamental work by Blackman et al in 1982 is known that in realization non-thermal effect of EHF EMF key role have Ca exchange which is involvement in great number of fundamental cell processes, such as Lipids exchange and nucleic acids metabolisms (Ziskin MC, 2006; Blackman et al (1982); Brini and Carafoli 2009)”. Sert et al. (2002) investigated the effect of 3 h/d exposure of the 0.8 mT magnetic field on the membrane fatty acids of testes and found that the fatty acid of the testis cell membrane phospholipids was decreased in the exposure group. It is similarly indicated that twenty minutes of 900 MHz RFR exposure (short term) do not alter the percentage of fatty acids in brain and testes (Dasdag et al. 2004, 2003). Recently, Oyarce et al. (2021) detected the main changes of some fatty acid, ergosterol and antioxidant activity of

metabolites in the fungus *Serpulohimantioide* exposed to 2.5 GHz Wi-Fi exposure. They observed an increment in the content of some fatty acids and ergosterol and finally stated that “Direct exposure to Wi-Fi radiation produces oxidative damage by modifying cellular components in the fungus *S. himantioide*” (Oyarce et al. 2021). If the World Health Organization (WHO) do not lead to put realistic educational and protective mechanisms, it is certain that human beings will continue to use wireless communication tools in incredible amounts and therefore will be exposed to more and more electromagnetic pollution with each passing day by ignoring health hazards. To draw attention to the subject, the aim of this study is to reveal the effects of long-term exposure of 2.4 GHz Wi-Fi exposure on some miRNAs such as rno-miR-22-3p, rno-miR-24-1-3p, rno-miR-132-3p, rno-miR-145-5p, rno-miR-181a-5p, rno-miR-186-5p, rno-miR-195-5p, rno-miR-219a-5p, rno-miR-221-3p, rno-miR-222-3p and fatty acids in brain tissue.

Materials and methods

Subjects and animal care

Sixteen Wistar Albino adult male rats were acquired from the Medical Science Application and Research Center of Dicle University. The rats were fed with standard pelleted food (TAVAS Inc., Adana, Turkey) in a standard Plexiglas cage. The average animal weight, which was determined to be 313 ± 2.5 g for each group at the beginning of the study, was 348 ± 28.8 g at the end of the study. Rats were equally divided into two groups such as sham exposed ($n = 8$) and exposure ($n = 8$) and kept on a 14/10 h light/dark schedule. During the study, the ambient temperature (22°C) and the relative humidity (45%) were maintained in the normal range for these animals. All animal procedures were in agreement with the Principles of Laboratory Animal Care and the rules of Scientific and Ethics Committee of Dicle University Health Research Center.

Exposure and field measurements

A pulsed signal generator, which could output Wi-Fi signals at 2.45 GHz, was used in the exposure system. Sham ($n = 8$) and experimental group animals ($n = 8$) were maintained free in their respective plexiglass cages to resume their daily activities without any restrictions. The experimental group, however, were exposed to 2.45 GHz RFR, about 50 cm below the transmitting antenna of the signal generator, every day (24 h/d) for one year.

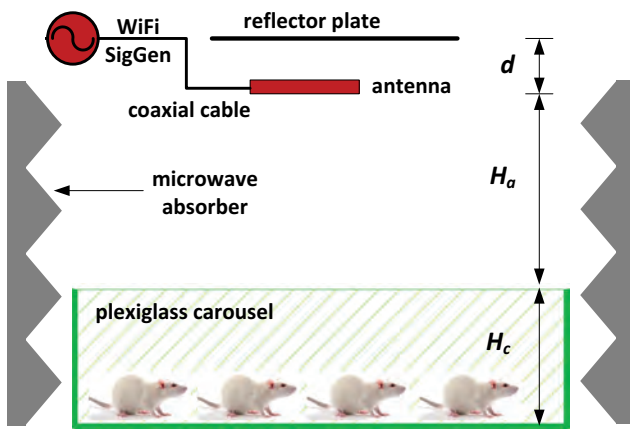


Figure 1. Experimental setup side view. The carousel length and width are 32 cm and 55 cm, respectively. $H_c = 20$ cm, $H_a = 50$ cm and $d = 2.5$ cm. Sixteen rats were free to move inside the carousel.

Similar to experimental group, animals in the control group lived 50 cm below the antenna of the generator (24h/d) for one year, except the generator was turned off. At the end of last exposure, the rats were intraperitoneally administered a combination of 6 mg/kg of 2% xylazine hydrochloride (Rompun) and 75 mg/kg ketamine hydrochloride (Ketalar) for anesthesia. Afterward, the brain of each rat were removed for the analysis.

Experiment setup is displayed in Fig. 1. Electromagnetic power density and the electrical field inside the Plexiglas cage were measured at several points using a field probe EMR 300 (NARDA, Pfullingen, Germany). To minimize coupling from unwanted radiation sources, four sides of the cage were covered with electromagnetic pyramidal absorber backed by metal sheets to isolate the test setup.

Specific absorption rate (SAR)

The Wi-Fi signal generator with a peak power of 100 mW was connected to a stub antenna, and the stub antenna was placed in front of a flat reflector to reduce outside radiation and to convey electromagnetic energy towards the Plexiglas carousel where rats were kept. The antenna was approximately 4λ away from the rats, where λ is the free space propagation wavelength at 2.45 GHz. With this separation, far-field conditions, which replicated typical Wi-Fi antenna radiation, were formed. At the far field of an antenna, electromagnetic energy can be modelled as a uniform plane wave. To calculate SAR within the rats, first, the electric field generated by the Wi-Fi signal source was measured at several locations of the carousel with a calibrated electric field probe. Then, these measured

electric field values were compared with the simulated electric field values where the whole experimental setup was formed in 3D electromagnetic field solver. CST Microwave Studio (CST AG, Germany) was used for electromagnetic field solutions and SAR calculations. Although CST seems similar to well-known finite-difference time domain solvers, it solves Maxwell's equation by the finite-integration technique employed on non-orthogonal grids. The rat voxel model used in CST was based on a computed tomography of a rat, and that model was used in SAR simulations. In SAR averaging calculations, the IEEE/IEC 62704 standard was used.

Lipid extraction and separation of lipid fractions

Total lipids were extracted with chloroform–methanol (2/1 v/v) (Folch et al. 1957). During the extraction process, autoxidation of unsaturated fatty acids was minimized by adding 50 μ L of 2% butylated hydroxytoluene (BHT) in chloroform (Stanley-Samuelson and Dadd 1983). Nonlipid contaminants were removed by extraction with 0.88% aqueous KCl (Folch et al. 1957). The extract was evaporated to dryness under a stream of nitrogen at room temperature. Thin layer chromatography (TLC) was used for separation of lipid classes. The petroleum ether/diethyl ether/glacial acetic acid (80/20/1, v/v) mobile phase was used to separate phospholipid and triacylglycerol fractions (Mangold 1969). For separation of phospholipid subclasses such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine, TLC plates were completely wetted in 1.8% boric acid prepared in 100% ethanol, dried for 5 min and activated for 15 min at 100°C. The lipid extracts were loaded on the plates and rapidly placed in the chromatography tank containing chloroform/ethanol/water/triethylamine (30/35/7/35, v/v/v/v) (Vaden et al. 2005). The TLC plates were then dried at room temperature. The PL classes were detected under UV light after having sprayed 2',7'-dichlorofluorescein on the TLC plates. The PL classes were then scrapped into separate test tubes, and their constitutive fatty acids were transmethylated through the addition of acidified (sulfuric acid, 1%) methanol for 2 h at 85°C¹¹. The resulting fatty acid methyl esters (FAMES) were extracted with hexane.

Chromatographic conditions

Fatty acid methyl esters were separated by capillary gas chromatography using a SHIMADZU GC 2010 PLUS equipped with a flame ionization detector (FID) and a fused silica capillary column (BP-21-Polyethylene

Glycol (terephthalic acid treated)) (J & W Scientific, Folsom, CA, USA, 25 m × 0.32 mm internal diameter × 0.25 µm film thickness). Helium was used as the carrier gas. Gas flow rates: helium, 0.5 mL min⁻¹; hydrogen, 30 mL min⁻¹; dry air, 400 mL min⁻¹. The temperature program used was as follows. The initial temperature (170°C) was kept for 8 min, raised to 190°C at a rate of 2°C min⁻¹ and kept at 190°C for 17 min and raised to 220°C at a rate of 10°C min⁻¹, and maintained at this temperature for 10 min. The injection and the detector temperatures were 250°C. The split ratio was 20/1. The FAMES identification and quantification were performed through comparison of retention times (identification) and peak areas (quantification) with a calibration solution composed of a known quantity of each pure FAME (Sigma-Aldrich Chemicals, St., Louis, MO, USA). The amount of fatty acids was given as a percentage.

Fatty acid analysis

Saturated Fatty Acids (SFAs) such as decanoic acid (10:0), lauric acid (12:0), myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (16:0), heptadecanoic acid (17:0), stearic acid (18:0) and eicosanoic acid (20:0); Monounsaturated Fatty Acid (MUFAs) such as palmitoleic acid (16:1 n-7), oleic acid (18:1 n-9) and eicosenoic acid (20:1 n-9); Polyunsaturated Fatty Acids (PUFAs) such as linoleic acid (18:2 n-6), α-linolenic acid (18:3 n-3), eicosadienoic acid (20:2 n-6), eicosatrienoic acid (20:3 n-6), arachidonic acid (20:4 n-6), eicosapentaenoic acid (20:5 n-3), docosapentaenoic acid (22:5 n-3) and docosahexaenoic acid (22:6 n-3) in total nineteen fatty acids were analysed in this study. In general, fatty acid compositions such as phospholipid (PL), triacylglycerol (TAG) and PL fractions such as phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE) were evaluated in this study.

RNA extraction

Total RNA was extracted from rat brain tissue using Tri-Reagent (Sigma).

Reverse transcriptase PCR reactions (RT-PCR)

Reverse transcriptase reactions contained 5 µl of extracted total RNA, 50 nM stem-loop RT primer, 5X RT buffer, 0.25 mM each of dNTPs, 50 units of modified M-MuLV Reverse Transcriptase (Thermo Scientific, Vilnius, Lithuania), 25 units of RiboLock RNase inhibitor (Thermo Scientific, Vilnius, Lithuania) and nuclease-

free water to a total reaction volume of 15 µl. The reaction was performed on an automated Thermal Cycler (Techne Flexigene, Cambridge, UK). RT-PCR conditions for 30 min at 16°C, 30 min at 42°C and 5 min at 85°C were used and then held at 4°C.

Quantitative-Comparative C_T (ΔΔC_T) real-time PCR

Quantitative-comparative C_T (ΔΔC_T) Real-time PCR was performed in an ABI Prism 7500 Real-Time PCR System (Applied Biosystems) using the SDS 2.0.6 software. The specific primers and fluorogenic ZNA™ probes (Paris et al. 2010) for the microRNAs were designed using Primer Express 3.0 software (Applied Biosystems) and are listed in Table 1. rno-miR-26b-5p was used as an endogenous control microRNA. The mixed RNA created from the sham group was used as a reference RNA sample. Primers and probes were purchased from Metabion International AG, D-82152 Martinsried/Deutschland. The 25 µl PCR included 3 µl of RT-PCR, 12.5 µl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nmol of each primer (Primer F and Universal Primer R) and 200 nmol TaqMan probe. The reactions were incubated in a 96-well plate of preincubation at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and at 60°C for 90 sec. All reactions were run in triplicate (Table 1).

Analyzed microRNA expression

In this study, we investigated effects of 2.4 GHz Wi-Fi exposure on some microRNAs such as rno-miR-22-3p, rno-miR-24-1-3p, rno-miR-132-3p, rno-miR-145-5p, rno-miR-181a-5p, rno-miR-186-5p, rno-miR-195-5p, rno-miR-219a-5p, rno-miR-221-3p and rno-miR-222-3p.

Statistical analyses

Statistical analyses were carried out using SPSS 18.0 software for Windows. The *t* test was used to determine whether there are any statistically significant differences between the means of control and Wi-Fi-exposed groups. The data in the tables were expressed as mean ± standard error (SE). The miRNA data were processed and analyzed using the statistical package STATISTICA 13 for Windows. Normality assumption of 2^{-ΔΔC_T} values was checked by the Shapiro-Wilk test. Since the assumption of normality was met, 2^{-ΔΔC_T} values were expressed as mean and standard deviation, the comparisons between groups were performed using an

Table 1. Primer/probe sequences of the miRNA analyzed by quantitative RT-PCR and Real-Time PCR.

miRNA Name	*miRNA Gene No	*miRNA Nucleotide Sequences No	Primer and Probe Sequences
rno-miR-26b-5p	100314229	NR-031831.1	RT 5'-GTCGTATGTCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACACCTAT-3' F 5'-GCCGCTTCAAGTAATTCAGG-3' PR 5'-FAM-TG(pdC)ATA(pdC)GA(pdC)A(pdC)CTATCC-ZNA4-BHQ-1-3'
rno-miR-22-3p	100314001	NR-031824.1	RT 5'-GTCGTATGTCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACACAGTT-3' F 5'-GCCGCAAGCTGCCAGTT-3' PR 5'-FAM-TG(pdC)ATA(pdC)GA(pdC)A(pdC)AGTT(pdC)T-ZNA4-BHQ-1-3'
rno-miR-24-1-3p	100314003	NR-031827.1	RT 5'-GTCGTATGTCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACCTGTTCC-3' F 5'-GCCGCTGGCTCAGTTCAG-3' PR 5'-FAM-TG(pdC)ATA(pdC)GA(pdC)CTGTTCTT-ZNA4-BHQ-1-3'
rno-miR-132-3p	100314029	NR-031878.1	RT 5'-GTCGTATGTCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACCGACCA-3' F 5'-GCCGCTAACAGTCTACAGCCA-3' PR 5'-FAM-TG(pdC)ATA(pdC)GACCGACCA-ZNA4-BHQ-1-3'
rno-miR-145-5p	100314036	NR-031891.1	RT 5'-GTCGTATGTCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACAGGGAT-3' F 5'-GCCGCTCCAGTTTTC-3' PR 5'-FAM-TG(pdC)ATA(pdC)GA(pdC)AGGGAT-ZNA4-BHQ-1-3'
rno-miR-181a-5p	100314195	NR-031926.1	RT 5'-GTCGTATGTCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACACTCAC-3' F 5'-GCCGCAACATTCAACGCTGTCG-3' PR 5'-FAM-TG(pdC)ATA(pdC)GA(pdC)ACT(pdC)ACCGA-BHQ-1-3'
rno-miR-186-5p	100314043	NR-031904.1	RT 5'-GTCGTATGTCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACAGCCCA-3' F 5'-GCCGCCAAGAATTCTCCTT-3' PR 5'-FAM-TG(pdC)ATA(pdC)GACAGCCCA-ZNA4-BHQ-1-3'
rno-miR-195-5p	100314281	NR-031912.1	RT 5'-GTCGTATGTCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACGCCAAT-3' F 5'-GCCGCTAGCAGCACAGAAAT-3' PR 5'-FAM-TG(pdC)ATA(pdC)GACGCCAAT-ZNA4-BHQ-1-3'
rno-miR-219a-5p	100314058	NR-031932.1	RT 5'-GTCGTATGTCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACAGAAAT-3' F 5'-GCCGCTGATTGTCCAAACG-3' PR 5'-FAM-TG(pdC)ATA(pdC)GA(pdC)AGAATTGC-ZNA4-BHQ-1-3'
rno-miR-221-3p	100314163	NR-031934.1	RT 5'-GTCGTATGTCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACGAAACC-3' F 5'-GCCGCAAGCTACATTGTCTGCTG-3' PR 5'-FAM-TG(pdC)ATA(pdC)GA(pdC)GAAA(pdC)CCA-BHQ-1-3'
rno-miR-222-3p	100314059	NR-031935.1	RT 5'-GTCGTATGTCAGTGCAGGGTCCGAGGTATTCGCACTGCATACGACCCAG-3' F 5'-GCCGCAAGCTACATCTGGCTA-3' PR 5'-FAM-TG(pdC)ATA(pdC)GA(pdC)ACC(pdC)AGTAGC-BHQ-1-3'
miR-Universal R			R 5'-GTGTCAGGGTCCGAGGTAT-3'

* <https://www.ncbi.nlm.nih.gov/gene>, Abbreviations: F: Forward, R: Reverse, PR: Probe, RT: Reverse Transcriptase Primer, pdC: Substitution of C-5 propynyl-dC (pdC) for dC is an effective strategy to enhance base pairing. Using these base substitutions, duplex stability and melting temperatures are raised by C-5 propynyl-C 2.8 per substitution, ZNA4: Zip Nucleic Acids: ZNA probes provide broad flexibility in assay design and represent an effective alternative to Minor Groove Binder (MGB)- and Locked Nucleic Acid (LNA)-containing oligonucleotides.

independent t-test and the significance values were adjusted for multiplicity using Benjamini-Hochberg adjustment (Benjamini and Hochberg 1995; Rai et al. 2012). The error-bar graph was used to represent data distribution of rno-miR-22-3p, rno-miR-24-1-3p, rno-miR-132-3p, rno-miR-145-5p, rno-miR-181a-5p, rno-miR-186-5p, rno-miR-195-5p, rno-miR-219a-5p, rno-miR-221-3p and rno-miR-222-3p variables according to the groups. Significant differences (two-tailed *P*) less than 0.05 were regarded as significant

Results

Phospholipid (PL) fatty acid composition in brain

2.4 GHz radiofrequency radiation increased docosapentaenoic acid (22:5 n-3) and decreased docosahexaenoic acid (22:6 n-3), which are members of n-3 Polyunsaturated Fatty Acids (n-3 PUFAs). Results are given in Figure 2 and Table 2.

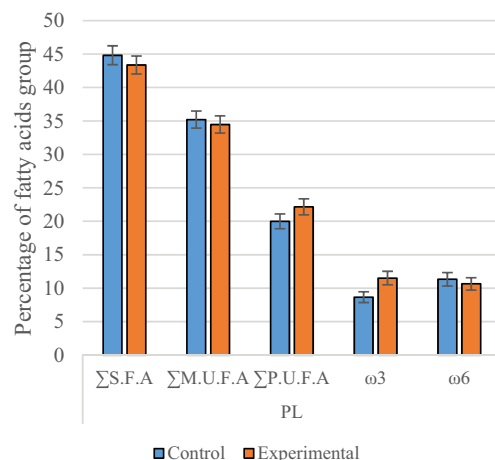


Figure 2. Percentage of fatty acid groups of brain phospholipid in control and experiment animals.

Triacylglycerol (TAG) fatty acid composition in brain

Since 2.4 GHz radiofrequency radiation increased linoleic acid (18:2 n-6), therefore, ΣPUFA and Σn-6 PUFA also increased. Results are shown in Figure 3 and Table 2.

Table 2. Fatty acid composition of brain PL, TAG and PL subclasses (%).

Fatty Acids	PL		TAG		PC		PI		PS		PE	
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
10:0 ^c	0.14 ± 0.01a	0.11 ± 0.03a	0.79 ± 0.06a	1.07 ± 0.16b	-	-	-	-	-	-	-	-
12:0	0.07 ± 0.02a	0.06 ± 0.02a	0.42 ± 0.03a	0.40 ± 0.02a	0.07 ± 0.02a	0.04 ± 0.02b	0.22 ± 0.02a	0.25 ± 0.02a	0.11 ± 0.03a	0.04 ± 0.02b	0.08 ± 0.02a	0.05 ± 0.03a
14:0	0.24 ± 0.01a	0.55 ± 0.05b	0.78 ± 0.05a	0.50 ± 0.07a	0.20 ± 0.01a	0.21 ± 0.01a	0.44 ± 0.03a	0.41 ± 0.15a	0.13 ± 0.04a	0.13 ± 0.04a	0.10 ± 0.04a	0.12 ± 0.03a
15:0	0.06 ± 0.02a	0.06 ± 0.02a	0.16 ± 0.01a	0.08 ± 0.02b	0.13 ± 0.04a	0.08 ± 0.02b	0.09 ± 0.01a	0.07 ± 0.02a	0.03 ± 0.02a	0.03 ± 0.02a	0.08 ± 0.02a	0.08 ± 0.02a
16:0	23.28 ± 1.22a	21.41 ± 1.12a	17.26 ± 1.17a	18.11 ± 1.18a	45.00 ± 1.45a	46.49 ± 1.47a	20.74 ± 1.13a	20.77 ± 1.13a	6.94 ± 0.69a	4.39 ± 0.41b	8.70 ± 0.85a	8.54 ± 0.81a
17:0	0.21 ± 0.01a	0.21 ± 0.01a	0.23 ± 0.01a	0.11 ± 0.03b	0.26 ± 0.02a	0.19 ± 0.01a	0.15 ± 0.03a	0.24 ± 0.02b	0.09 ± 0.01a	0.20 ± 0.01b	0.29 ± 0.02a	0.18 ± 0.01a
18:0	20.49 ± 1.18a	20.51 ± 1.22a	5.56 ± 0.51a	3.82 ± 0.20b	12.99 ± 1.02a	13.57 ± 1.03a	25.34 ± 1.27a	25.27 ± 1.33a	39.84 ± 1.33a	47.84 ± 1.49b	25.39 ± 1.27a	24.39 ± 1.14a
20:0	0.31 ± 0.02a	0.47 ± 0.04a	0.22 ± 0.01a	0.13 ± 0.04a	0.14 ± 0.01a	0.14 ± 0.01a	0.38 ± 0.02a	0.41 ± 0.15a	0.38 ± 0.02a	0.50 ± 0.07b	0.11 ± 0.03a	0.13 ± 0.04a
ΣS.F.A	44.82 ± 1.42a	43.37 ± 1.34a	25.44 ± 1.13a	24.22 ± 1.24a	58.78 ± 1.57a	60.74 ± 1.62a	47.37 ± 1.48a	47.42 ± 1.46a	47.51 ± 1.44a	53.14 ± 1.52b	34.73 ± 1.30a	33.50 ± 1.29a
16:1 n-7	0.44 ± 0.03a	0.44 ± 0.03a	0.90 ± 0.09a	0.67 ± 0.07a	0.33 ± 0.02a	0.33 ± 0.02a	0.96 ± 0.10a	0.98 ± 0.09a	0.11 ± 0.03a	0.04 ± 0.02b	0.15 ± 0.03a	0.24 ± 0.02b
18:1 n-9	33.53 ± 1.32a	32.26 ± 1.30a	58.89 ± 1.55a	57.32 ± 1.63a	30.29 ± 1.44a	30.30 ± 1.19a	18.88 ± 1.16a	18.97 ± 1.08a	31.61 ± 1.19a	27.07 ± 1.40a	26.33 ± 1.13a	27.21 ± 1.27a
20:1 n-9	1.22 ± 0.33a	1.77 ± 0.12a	0.31 ± 0.02a	0.13 ± 0.04b	0.89 ± 0.29a	0.63 ± 0.17b	0.44 ± 0.03a	0.38 ± 0.02a	1.36 ± 0.11a	1.62 ± 0.17a	3.81 ± 0.35a	4.68 ± 0.44a
ΣM.U.F.A	35.20 ± 1.28a	34.47 ± 1.29a	60.10 ± 1.63a	58.12 ± 1.58a	31.51 ± 1.30a	31.26 ± 1.35a	20.29 ± 1.22a	20.33 ± 1.29a	33.08 ± 1.37a	28.73 ± 1.30a	30.29 ± 1.14a	32.14 ± 1.35a
18:2 n-6	3.58 ± 0.32a	2.17 ± 0.20a	12.73 ± 1.04a	16.22 ± 1.08a	1.88 ± 0.13a	0.74 ± 0.16b	1.88 ± 0.13a	1.93 ± 0.17a	3.42 ± 0.15a	0.40 ± 0.02b	1.28 ± 0.12a	0.71 ± 0.06b
18:3 n-3	0.11 ± 0.03a	0.03 ± 0.02b	0.56 ± 0.03a	0.78 ± 0.05a	0.02 ± 0.01a	0.01 ± 0.01a	0.13 ± 0.04a	0.14 ± 0.01a	0.10 ± 0.04a	0.01 ± 0.01b	0.06 ± 0.02a	0.09 ± 0.01a
20:2 n-6	0.14 ± 0.01a	0.16 ± 0.01a	0.15 ± 0.03a	0.03 ± 0.02b	0.10 ± 0.04a	0.05 ± 0.03b	0.17 ± 0.06a	0.20 ± 0.01a	0.10 ± 0.04a	0.13 ± 0.04a	0.21 ± 0.01a	0.25 ± 0.02a
20:3 n-6	0.16 ± 0.01a	0.22 ± 0.02a	0.01 ± 0.01a	0.003 ± 0.01b	0.11 ± 0.03a	0.06 ± 0.02b	0.19 ± 0.01a	0.19 ± 0.01a	0.19 ± 0.01a	0.32 ± 0.02b	0.23 ± 0.01a	0.32 ± 0.02a
20:4 n-6	7.45 ± 0.77a	8.10 ± 0.71a	0.66 ± 0.22a	0.39 ± 0.03b	4.90 ± 0.38a	4.68 ± 0.44a	24.32 ± 1.27a	24.05 ± 1.10a	3.13 ± 0.33a	3.62 ± 0.32a	14.96 ± 1.05a	14.81 ± 1.05a
20:5 n-3	0.36 ± 0.03a	0.34 ± 0.03a	0.08 ± 0.22a	0.03 ± 0.02b	0.11 ± 0.03a	0.14 ± 0.01a	0.73 ± 0.06a	1.11 ± 0.19b	0.56 ± 0.04a	1.23 ± 0.30b	0.02 ± 0.01a	0.24 ± 0.02b
22:5 n-3	0.43 ± 0.04a	9.07 ± 0.91b	0.05 ± 0.03a	0.04 ± 0.02a	0.04 ± 0.02a	0.11 ± 0.03b	1.30 ± 0.22a	1.18 ± 0.11a	0.35 ± 0.02a	0.40 ± 0.02a	0.17 ± 0.06a	0.32 ± 0.02b
22:6 n-3	7.76 ± 0.55a	2.08 ± 0.12b	0.22 ± 0.02a	0.19 ± 0.01a	2.55 ± 0.12a	2.21 ± 0.29a	3.62 ± 0.32a	3.47 ± 0.34a	11.57 ± 0.97a	12.02 ± 1.02a	18.04 ± 1.09a	17.64 ± 1.07a
ΣP.U.F.A	19.99 ± 1.10a	22.16 ± 1.20a	14.47 ± 1.17a	17.66 ± 1.09a	9.71 ± 0.84a	8.00 ± 0.80a	32.34 ± 1.33a	32.26 ± 1.30a	19.42 ± 1.09a	18.12 ± 1.09a	34.98 ± 1.31a	34.37 ± 1.33a
Σω3	8.66 ± 0.81a	11.51 ± 1.01a	0.92 ± 0.10a	1.03 ± 0.12a	2.72 ± 0.21a	2.46 ± 0.30a	5.78 ± 0.56a	5.89 ± 0.56a	12.57 ± 1.02a	13.65 ± 1.03a	18.29 ± 1.07a	18.29 ± 1.08a
Σω6	11.33 ± 1.01a	10.65 ± 0.93a	13.55 ± 1.14a	16.63 ± 1.13a	6.99 ± 0.63a	5.53 ± 0.15a	26.56 ± 1.32a	26.36 ± 1.13a	6.84 ± 0.62a	4.48 ± 0.42a	16.69 ± 1.16a	16.08 ± 1.06a
ω3/ω6	0.76	1.08	0.07	0.06	0.39	0.44	0.22	0.22	1.84	3.05	1.10	1.14

Values are provided as mean ± SE; SFA saturated fatty acid, MUFA monounsaturated fatty acid, PUFA polyunsaturated fatty acid. Significant differences at a level of $p < 0.05$ are designated by 'a' and 'b' for PC, PE, PI and PS; a and b for PC, PE, PI and PS in control and experiment animals

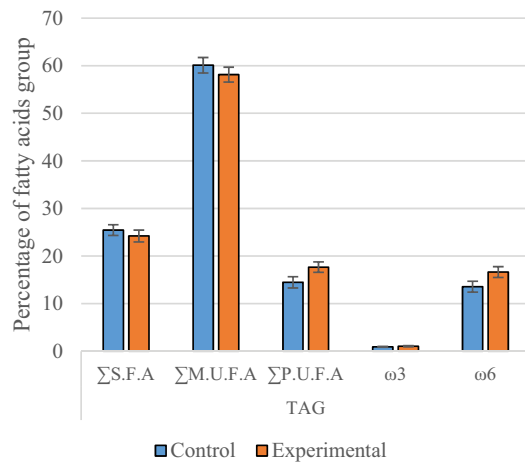


Figure 3. Percentage of fatty acid groups of brain triacylglycerol in control and experiment animals.

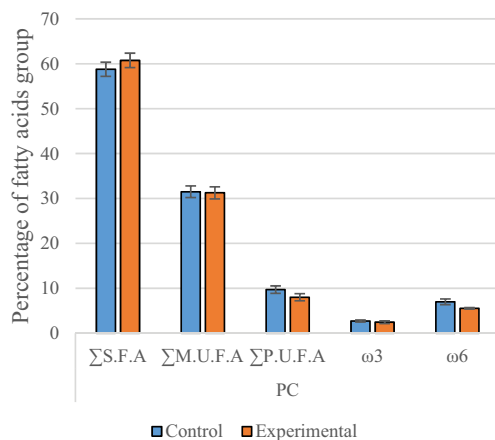


Figure 4. Percentage of fatty acid groups of brain phosphatidylcholine in control and experiment animals.

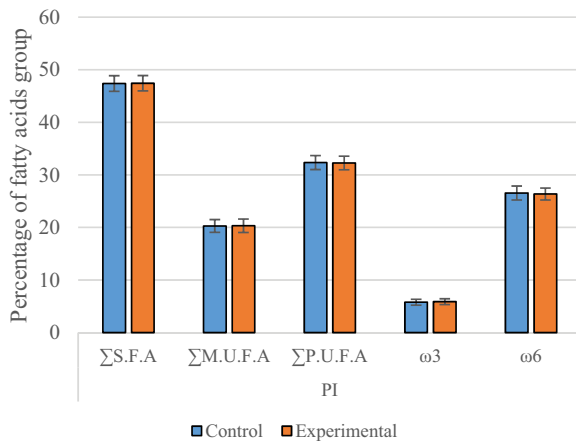


Figure 5. Percentage of fatty acid groups of brain phosphatidylinositol in control and experiment animals.

PL subclasses of fatty acid composition in brain

Phosphatidylcholine (PC) fatty acid composition

Any differences were not observed between the 2.4 GHz Wi-Fi exposure group and sham animals in terms of PC in brain. Results are given in [Figure 4](#) and [Table 2](#)

Phosphatidylinositol (PI) fatty acid composition

Any differences were not observed between the 2.4 GHz Wi-Fi exposure group and sham animals in terms of PI fatty acid composition in brain. Results are given in [Figure 5](#) and [Table 2](#).

Phosphatidylserine (PS) fatty acid composition

2.4 GHz Wi-fi increased the percentage of 18:0, which is the member of Saturated Fatty Acids (SFAs). However, 2.4 GHz Wi-fi decreased the percentage of 18:1 n-9, which is a member of Monounsaturated Fatty Acids (MUFAs) and 18:2 n-6 which is the member of n-6 Polyunsaturated Fatty Acid (n-6 PUFAs). Results are given in [Figure 6](#) and [Table 2](#).

Phosphatidylethanolamine (PE) fatty acid composition

Any differences were not observed between the 2.4 GHz Wi-Fi exposure group and sham animals in terms of PE fatty acid composition in brain. Results are given in [Figure 7](#) and [Table 2](#).

In summary, 2.4 GHz Wi-Fi exposure has potential to change the fatty acid composition of the membrane lipids phospholipid and phosphatidylserine with triacylglycerol, the storage lipid in brain.

The percentage of fatty acids in sham and exposed groups is given in [Table 2](#).

microRNA expression results

As it is stated above, we evaluated the effect of 2.4 GHz RFR exposure on ten miRNAs. At the end of the study, we observed that only miR_181a_5p increased by 2.4 GHz Wi-Fi exposure ([Table 3](#)).

The results of this study showed that the long-term 2.4 GHz Wi-Fi exposure increased expression of rno-miR-181a-5p ($p = 0, 007743$), while other microRNAs investigated in this study were not altered ($p > .05$).

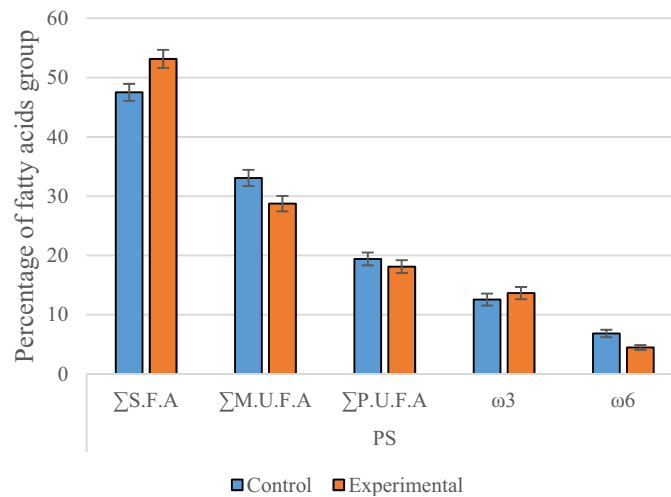


Figure 6. Percentage of fatty acid groups of brain phosphatidylserine in control and experiment animals.

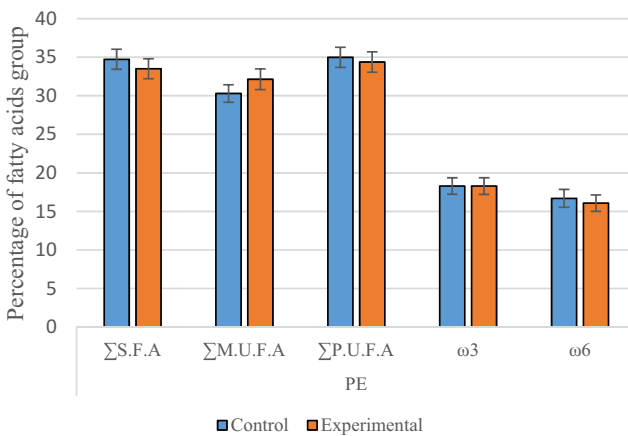


Figure 7. Percentage of fatty acid groups of brain phosphatidylethanolamine in control and experiment animals.

Table 3. Results of microRNAs expression investigated in this study.

microRNAs	Sham Mean	Std. Dev. ±	2.4 GHz Mean	Std. Dev. ±	p
rno-miR-22-3p	0.678560	0.296382	0.756667	0.126383	0.529866
rno-miR-24-1-3p	0.649322	0.230953	0.633689	0.134501	0.877750
rno-miR-132-3p	1.073392	0.534161	1.361540	0.216013	0.206306
rno-miR-145-5p	1.051213	0.632334	1.623565	0.599016	0.096452
rno-miR-181a-5p	0.483891	0.140160	0.666881	0.066802	0.007743
rno-miR-186-5p	0.499652	0.159833	0.522221	0.108539	0.757931
rno-miR-195-5p	0.593268	0.171782	0.521081	0.150385	0.405576
rno-miR-219a-5p	0.271842	0.099724	0.253847	0.360437	0.908345
rno-miR-221-3p	0.281981	0.142452	0.315691	0.085829	0.595539
rno-miR-222-3p	0.427805	0.263579	0.547197	0.88440	0.338093

SAR (Specific Absorption Rate) result

Brain, cerebral fluid, blood arteries and blood of the simulated rat are shown in Figure 8 a. Coronal SAR distribution for 10 g averaging and saggital SAR distribution for 1 g averaging are shown in Figure 7 b and c. Maximum SAR levels in those regions were 1543

μW/kg and 655 μW/kg, for 1 g and 10 g averaging, respectively. Whole body total SAR and maximum point SAR were calculated to be 328 μW/kg and 7197 μW/kg, respectively.

Discussion

It is reported that “The discovery of microRNAs has revealed an unexpected and spectacular additional level of fine tuning of the genome, and how genes are used again and again in different combinations to generate the complexity that forms, for instance, the brain”. Although, microRNAs are abundantly expressed in the brain, relatively little is known about the multiple functions of these RNA molecules in the nervous system. MicroRNA pathways play major roles in the proliferation, differentiation, function and maintenance of neuronal cells. Several intriguing studies have linked microRNAs as major regulators of the neuronal phenotype and have implicated specific microRNAs in the regulation of synapse formation and plasticity. Dysfunction of microRNA pathways is also slowly emerging as a potential important contributor to the pathogenesis of major neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease” (De and Christen 2010; He’bert et al. 2010). It is also stated that miRNA research has mainly focused on cancer (He’bert et al. 2010). As it is known, miRNAs were recently very popular; however, the study performed to reveal effects of RFR exposure on miRNAs is very limited as stated above. Under radiofrequency radiation, the relationship between RFR and various diseases, especially cancer, is underestimated. For instance, Dasdag et al. (2020) summarized that “The

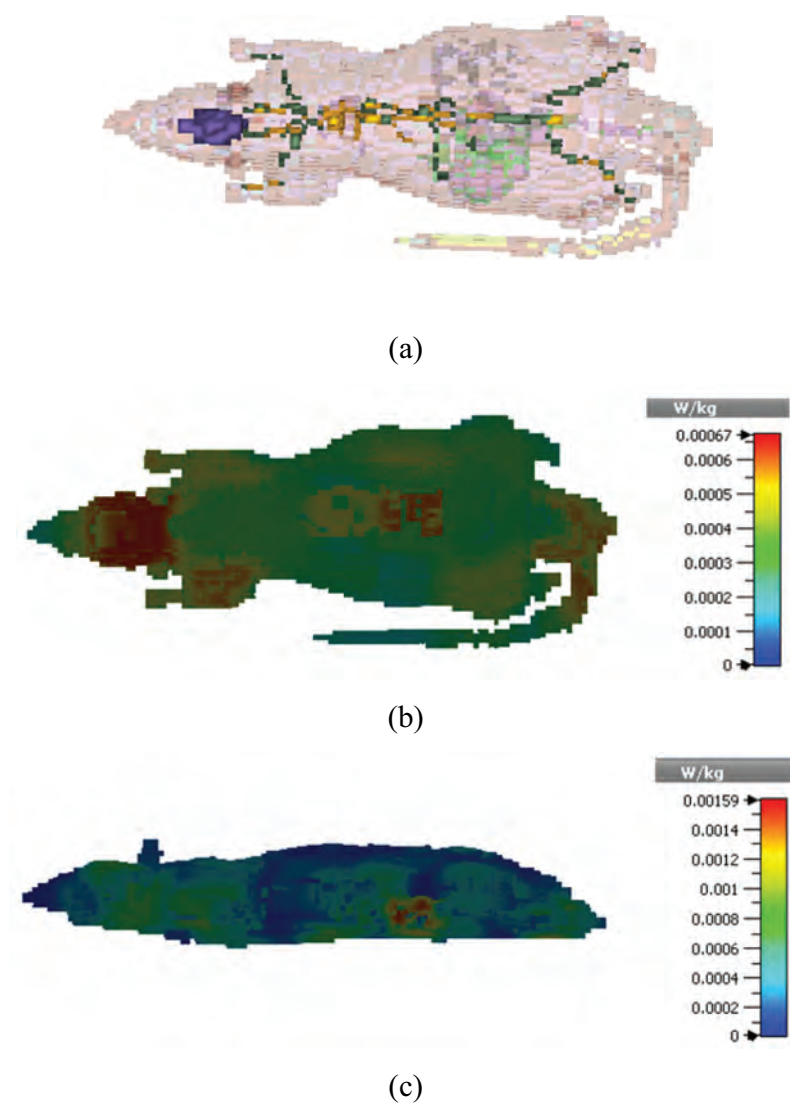


Figure 8. Rat SAR simulations: a) SAR model, b) coronal cut for 10 g averaging and c) saggital cut for 1 g averaging.

Table 4. Changes in fatty acid composition triggered by Wi-Fi exposure.

Fatty Acid	PL	TAG	PC	PI	PS	PE
10:0		Increased				
12:0			Decreased		Decreased	
14:0	Decreased					
15:0		Decreased				
16:0					Decreased	
17:0		Decreased			Increased	
18:0		Decreased			Increased	
20:0					Increased	
ΣS.F.A					Increased	
20:1 n-9		Decreased	Decreased			
18:2 n-6			Decreased		Decreased	Decreased
18:3 n-3	Decreased				Decreased	
20:2 n-6		Decreased				
20:3 n-6		Decreased			Increased	
20:4 n-6		Decreased				
20:5 n-3		Decreased		Increased	Increased	Increased
22:5 n-3		Increased	Increased			Increased
22:6 n-3	Decreased					

results of the study investigating the relationship between Alzheimer's disease (AD) and electromagnetic fields were still contradictory". Whereas many researchers suggest that some electromagnetic radiation has prepared the ground for AD, others claim that RF radiation has a positive effect on the disease in question" (Lin et al. 2021). Therefore, it may be too late when the evidence between certain diseases and electromagnetic pollution is established in "the future.

The results of our previous studies, which are in a limited number of studies examining the relationship between RFRs and microRNAs, revealed that this radiation has potential to predispose to different diseases. Although the results obtained from animal studies must be taken into consideration by the relevant persons or organizations, there is a need for comprehensive studies. However, the results of our previous studies on microRNAs indicated that 900 MHz RFR emitted from mobile phones has potential to decrease rno-miR107 expression and increase rno-miR-145-5p in brain. However, we also observed that miR-107 expression was 3.3 times and miR-106b-5p expression was 3.65 times lower in the 2.4 GHz Wi-Fi exposure group. The relation between the alteration in rno-miR107, rno-miR-145-5p and miR-106b-5p and some diseases has been discussed in detail in our previous studies. In these studies, we observed that rno-miR107 is more sensitive to both RFR radiation more than two different miRNAs (Dasdag et al. 2015a, 2015b; Dasdag et al. 2019).

As it is discussed above, we investigate the effect of 2.4 GHz Wi-Fi exposure on ten different microRNAs such as rno-miR-22-3p, rno-miR-24-1-3p, rno-miR-132-3p, rno-miR-145-5p, rno-miR-181a-5p, rno-miR-186-5p, rno-miR-195-5p, rno-miR-219a-5p, rno-miR-221-3p and, rno-miR-222-3p in this study. However, we observed that 2.4 GHz RFR only increased rno-miR-181a-5p expression, which is stated that "miR-181a-5p increased the chemical sensitivity of HS578T cells to cisplatin by inhibiting VDR to promote autophagy" (Lin et al. 2021). Therefore, we can state that our previous studies supported the results of this study in terms of indicating the effects of RFRs on miRNA. miR-181a-5p was highly expressed in serum of acute ischemic injury patients" (Song et al., 2021). On the other hand, Liu et al. stated that "miR-181a-5p is a miRNA shown to play an essential role in multiple cancers, such as hepatic cancer, non-small cell lung cancer and human diseases, such as osteoarthritis. The interaction between lncRNA CCAT1 and miR-181a-5p has been demonstrated in endometrial carcinoma" (Liu et al. 2020).

As a result, we can say that it should be focused on the diseases associated with rno-miR107, rno-miR-145-5p, miR-106b-5p and rno-miR-181a-5p expressions in humans exposed to long-term RFR exposure. Illumination of these relationships may also create opportunities to develop new approach various diseases.

As it is known, we also evaluated interaction between 2.4 GHz Wi-Fi exposure and membrane fatty acid of brain tissue in this study. Our previous studies indicated that short-term exposure of RFR does not have potential to alter membrane fatty acids in different tissues. Since our previous studies represent short-term exposure, we have no chance to compare the results of them with the data obtained in this study (Dasdag et al. 2004, 2003; Sert et al. 2002). However, the data of Oyarce et al. (2021) indicated that Wi-Fi exposure has potential to cause an increment in the content of some fatty acids. This means that there is an agreement between the results of this study and Oyarce's data (Oyarce et al. 2021). On the other hand, Fragopoulou et al. (2018) observed some alteration in membrane fatty acids in brain and stated that "The observed changes indicate a membrane remodeling response of the tissue phospholipids after nonionizing radiation exposure, reducing SFA and EPA, while increasing MUFA residues". They also claimed that RFR radiation induces hippocampal lipidome and transcriptome changes that may explain the brain proteome changes and memory deficits". In Fragopoulou's study, some fatty acids such as 16:0, 20:5 ω -3 and SFA decreased, while 16:1 and 18:1-9c increased by RFR exposure (Fragopoulou et al. 2018).

In our study, we also observed that 2.4 GHz Wi-Fi exposure altered some fatty acids. 2.4 GHz exposure caused changes in PL and TAG percentages, but changes in TAG were found to be more pronounced. However, PS, which is subclasses of PL, is affected by RFR more than other subgroups. It is reported that "Phosphatidylserine is subclasses called a phospholipid. It covers and protects the cells in your brain and carries messages between them. Phosphatidylserine plays an important role in keeping the mind and memory sharp. Animal studies suggest that the level of this substance in the brain decreases with age" (Burr and Burr 1930). Therefore, our results on the changes in the fatty acid composition of phosphatidylserine gain an importance because 2.4 GHz Wi-Fi exposure decreased percentage of linoleic acid (18:2 n-6). Linoleic acid (LA, 18:2 n-6) is an essential n-6 polyunsaturated fatty acid (PUFA) required for normal growth and development. However, little is known about how this increase in dietary LA affects the brain. It is mainly viewed as an essential precursor to arachidonic acid (20:4 n-6, AA), which is

important for neurodevelopment and other physiological processes (Brenna 2016). A summary of fatty acid alteration triggered by Wi-Fi exposure is given in Table 4. However, although the results we obtained in this study are consistent with the results of Fragopoulou et al. (2018), ours are more pronounced. The reason of this situation sourced from the exposure setup and duration differences. For instance, we used a generator to standardize the exposure and investigated long-term/chronic effects of RFRs by exposing animals during day (24 hours) for one year. Whereas they exposed animals to 1800 MHz RFR exposure for 2 hours emitted from a commercial mobile phone is investigated. Since RFR exposure cannot be standardized because of some physical characteristics, using a mobile phone in the study like this is not acceptable. Although this exposure setup was acceptable when studies in this field were started first, they are not realistic today.

On the other hand, It is interestingly reported that “electronically generated electromagnetic fields are coherent, producing very high electric and magnetic forces, which act on the voltage sensor of voltage-gated calcium channels to produce increases in intracellular calcium $[Ca^{2+}]_i$ ” (Pall. 2022). Blaustein and Lederer reported that “In controlling of intracellular Ca homeostasis have key role cGMP-dependent Ca efflux from the cell, which realized by Na/Ca exchange in forward (F) mode and Ca-pump in cell membrane and cAMP-Ca exchange in/Ca exchange in reverse (R) mode”. They also stated that “the discovery of multiple Na^+/Ca^{2+} exchanger genes and gene products, and multiple splice variants, as well as exchangers with different coupling ratios [i.e., the 3 $Na^+:1Ca^{2+}$ exchanger a Na^{2+}/Ca^{2+} exchanger and the 4 $Na^:(1Ca^{2+} + 1K^+)$ exchanger] has added to the complexity. We must even consider the possibility that some cells may express more than one Na^+/Ca^{2+} exchanger [or $Na^+/(Ca^{2+} + K^+)$ gene product or alternatively spliced isoform] and that these exchangers may perform different functions” (Blaustein and Lederer, 1999). By Ayrapetyan work have shown that in cell membrane have two family of quantum-sensitive receptors through which the non-thermal effect of non-ionizing radiation on cells are realized they are soluble guanylate cyclase (sGC) and G proteins family of receptors the activation of which leads to stimulate the cAMP-dependent RNa/Ca exchange (Ayrapetyan, 2018). Therefore we hypothesis that the non-thermal radioaction-induced changes of physicochemical properties of circulating blood by stimulation of quantum-sensitive membrane receptors induced $[Ca]_i$ changes could be primary mechanism for modulation of lipids metabolism and RNA metabolism. To check this hypothesis will be the aim of future works.

Conclusion

The results of this study revealed that long-term and chronic exposure of 2.4 GHz Wi-Fi radiation increased rno-miR-181a-5p expression and changed the percentage of some fatty acids and its subclasses. Therefore, it should be kept in mind that the radiofrequency radiations emitted by various internet providers, mobile phones and other wireless communication devices that we are exposed to throughout the day have the potential risk. Being cautious is the most reasonable approach. The uncontrolled exposure of RFRs, whose use and diversity have reached incredible levels with each passing day and which are increasing in the future, may be paving the way for many diseases that we cannot connect with today. Therefore, parents have to pay more attention to this risk to protect themselves and especially children and adolescent.

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