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# APOPTOSIS RESULTED FROM RADIOFREQUENCY RADIATION EXPOSURE OF PREGNANT RABBITS AND THEIR INFANTS

GOKNUR GULER, ELCIN OZGUR, HIKMET KELES[1], ARIN TOMRUK, SEVIL ATALAY VURAL[2], AND NESRIN SEYHAN

Department of Biophysics, Gazi Non-Ionizing Radiation Protection Center (GNRK), Medical Faculty, Gazi University, Ankara, 06500, Turkey
[1]Department of Pathology, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03200, Afyon, Turkey
[2]Department of Pathology, Faculty of Veterinary Medicine, Ankara University, 06110, Ankara, Turkey

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## Abstract

The purpose of this study was to reveal the apoptotic cell formation, using histopathological and immunohistochemical methods, in non-pregnant and pregnant New Zealand White rabbits, and in offspring of the pregnant group exposed to GSM modulated signal in 1,800 MHz frequency. Apoptotic cells were detected in the brain, eyes, kidneys, liver, lung, heart, and spleen by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) staining. Histopathological changes were observed in the examined organs. TUNEL positivity was seen in the brain (group VI) and eyes (groups IV and VI). In groups I, II, III, and V, the positivity was lesser than 5% and was not taken into account.

**Key words:** rabbits, pregnancy, radiofrequency radiation, apoptosis.

Over the last decades, electromagnetic pollution ("environmental electromagnetic fields") has been one of the main environmental health problems associated with man-made sources such as high power lines, transformers, base stations, TV and radio transformers, radars, microwave ovens, diathermy and magnetic resonance imaging units. Although the relationship between environmental electromagnetic fields and their adverse health effects are still unclear, scientists have added it to the list of potential sources of adverse health effects.

Electromagnetic fields with the frequencies of 100 kHz to 300 GHz are usually named as radio-frequency radiation (RFR). RFR resulted from a wide and fast-growing use of wireless technologies especially mobile phones and their base stations has led to an increase in public and scientific attention. WHO has identified possible adverse effects of mobile phones on the developing tissues as a major research priority.

According to the report of the Advisory Committee in recent WHO workshop in Istanbul, Turkey, hosted by the Biophysics Department of Gazi University in 2004, it is recommended to study the effect of radiofrequency (RF) field exposure on the developing tissues in animal models. To clarify the possible link between RFR and health effects, scientists have been investigating this problem more than 20 years. Most of the recent epidemiological and experimental (*in vivo/in vitro*) studies have indicated that acute or chronic exposure in different frequency ranges may alter biological responses including cell cycle (4), cell proliferation (5, 20, 37), apoptosis (26, 42), and DNA damage (7, 21-23, 35).

The cell death or the development of cancer associated with RFR should be taken into account since it leads to the structural changes in DNA or chromosomes of somatic cells that might be inherited from generation to generation (38). Programmed cell death known as apoptosis is expressed in morphological changes that are observed in cell proliferation, differentiation, and tumour cell depletion. These morphological changes have been regulated by a number of well-characterised genes. A number of physical, biological, and toxic agents can lead to apoptotic deaths (13, 17). Apoptosis is considered to be different from degenerative death or necrosis due to its different mechanism and its biological importance (16). In the apoptosis, the death process has been conducted directly by the cell, whereas cells affected by external agents have led to the death, which is signalled by irreversible changes in the nucleus and in the cytoplasm (25). Apoptosis is particularly important in the development

of the nervous system and in the development and effective functioning of the immune system (12).

Various methods, such as the agarose gel electrophoresis using extracted DNA, staining methods using fluorescence stain, flow cytometry, detection of apoptosis-related genes and their products (Bcl-2, Fas, Fas ligand p53, members of caspase family), immunohistochemistry, *in situ* hybridisation, Western blotting, and RT-PCR, and terminal deoxynucleotidyl transferase (TdT)- mediated dUTP-biotin nick end-labelling (TUNEL) method were developed and applied for apoptosis detection. The TUNEL method is suitable for analysing the topographical distribution of cells with free Y-OH ends of DNA, because a condensed chromatin contains many fragments of DNA (30). The most specific assay is perhaps the oldest, the detection of nuclear shape changes in the early stages of apoptosis. In combination with other methods, this morphological interpretation usually allows a relatively accurate interpretation of apoptosis. For tissue sections, many investigators recommend labelling of DNA strand breaks (ISNT, TUNEL, anti-SS DNA) together with analysis of nuclear morphology (39). A widely used method for identifying dying cells is the TUNEL assay (10).

The present study can be considered as unique to reveal the apoptotic cell formation by using histopathological and immunohistochemical methods in non-pregnant and pregnant rabbits and in their newborns exposed to GSM modulated signal.

## Material and Methods

**Animals.** Eighteen non-pregnant and 18 pregnant 13-month-old New Zealand White rabbits obtained from the Laboratory of Animal Breeding and Experimental Researches Centre of Gazi University were used in the experiment. The experimental protocol was reviewed and approved by the Laboratory Animal Care Committee of Gazi University. The rabbits were housed under the same conditions at temperature and humidity controlled room (20 ±1°C, 50 ±10% relative humidity) and 14-16 h light/dark cycle. Except exposure periods, tap water and standard pelleted feed were provided *ad libitum*. For breeding, virgin female rabbits were placed individually with male rabbit. After mating, their pregnancies were verified by abdominal palpation 10 d later. Pregnant and non-pregnant rabbits were adapted for 5 d to the laboratory conditions before the experiment. During these days, quality controls were conducted to verify that rabbits used in this experiment were healthy. Pregnant rabbits, between $15^{th}$ and $22^{nd}$ d of gestation, and non-pregnant rabbits were exposed to RFR after the adaptation period. Since placing more than one animal in a cage would create a stress factor, only one animal was placed in each cage during each RFR exposure period. The rectal temperatures were measured in all rabbits by digital thermometer (Elite, Turkey) before and after the exposure. After birth (max. 2 d), a newborn from each litter was selected randomly and decapitated immediately.

**Exposure level and quality control.** GSM like signals in 1,800 MHz frequency were formed by using a signal generator (Agilent Technologies 8648C, 9 kHz-3.2 GHz) with the integrated pulse modulation unit and horn antenna (Schwarzbeck, Doppelsteg Breitband Horn antenna BBHA 9120 L3F, 0.5 - 2.8 GHz) in a shielded room. The generated power was controlled by a spectrum analyser (Agilent Technologies N9320A, 9 kHz - 3 GHz) integrated to the signal generator. The signals were amplitude modulated by rectangular pulses with a repetition frequency of 217 Hz and a duty cycle of 1:8 (pulse width 0.576 ms), corresponding to the dominant modulation component of GSM.

RFR generator provided 20 dBm (0.1 W) power during the exposure period. The signal controlled by means of spectrum analyser connected to the signal generator, also NARDA EMR 300 and type 26.1 probe was used for the measurement of the output radiation. Measurements were taken during the experiment and the data were saved to the computer connected to the device *via* fiber optic cable. The evaluated data was 14 ±0.5 V/m.

**Experimental design.** Non-pregnant and pregnant rabbits were randomly divided into four equal groups: group I (non-pregnant control) - each rabbit was kept in plexiglass cage under experimental setup for 15 min/d during 7 d but device was switched-off; group II (non-pregnant RFR-exposed,) - rabbits were exposed individually to 1,800 MHz GSM-like RFR for 15 min/d during 7 d; group III (pregnant control) -rabbits were kept in plexiglas cage under experimental setup for 15 min/d during 7 d but device was switched-off; group IV (pregnant RFR-exposed) - rabbits were exposed individually to 1,800 MHz GSM-like RFR for 15 min/d during 7 d. Newborns (n=18) were divided into two equal groups: group V - newborns of group III females and group VI - newborns of group IV females.

**Euthanasia and histopathological examination**. The day after the last exposure, all rabbits were anesthetised by the injections of ketamine (35 mg/kg, i.m.) and xylazine (5-10 mg/kg, i.m.) and killed by cervical decapitation.

The brain, eyes, liver, kidneys, lung, heart, and spleen were removed, fixed in 10% buffered formalin, processed, and embedded in paraffin. The sections were cut at 5 μm and stained with haematoxylin-eosin (HE).

**Assessment of apoptotic cells.** Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining using a commercial ready-to-use kit (*in situ* cell death detection kit, POD, Roche, Germany). The procedure and control stainings were carried out according to the manufacturer's instruction. After deparaffinisation and rehydration, tissue sections were digested with proteinase K (20 μg/mL, 30 min) and methanol with 3% hydrogen peroxide in PBS (5 min). Then the sections were incubated in a humidified chamber in 200 μl of TUNEL mixture (TdT and label solution) at $37^0$C for 60 min and with POD converter at $37^0$C for 30 min. The sections were then treated with 3-amino-9-ethyl-

carbasole (AEC) as a chromogen (Dako, USA) for 5 min, washed with PBS (pH 7.4), and counterstained with Mayer's haematoxylin.

TUNEL sections were blindly examined by two pathologists under light microscope (Leica DM 4000B) interfaced with a camera (Leica, DFC 80). TUNEL positivity was evaluated by a semi-quantitative scoring system according to Xu *et al*. (40) with minor modifications. Ten different fields on each slide were examined at high magnification. The intensity of staining was scored as negative (-), mild (+), moderate (++), and severe (+++). The extent of staining was scored as - (0%-5%), + (6%-25%), ++ (26%-50%), and +++ (51% and higher) according to the percentage of positively stained cells. Each field was graded according to the score and then the total score was divided by ten. This way the average score was calculated for each slide.

## Results

Analysed and scored results of histopathological and TUNEL methods are presented in Tables 1-3. These results varied among experimental groups and among animals in each group. Histopathologically; hyperaemia, haemorrhage, neuronal necrobiosis, clarity of Nissl substance, gliosis (Fig. 1), and mononuclear cells in perivascular areas were detected in the brain. Oedema, epithelial degeneration (Fig. 2), and mononuclear cells in cornea, oedema, and hyperaemia in the *corpus ciliare* were seen in the eyes. Passive hyperaemia, hepatocyte degeneration of various degree (Fig 3) and mononuclear cell clusters in sinusoids and/or periportal areas were recorded in the liver. Hyperaemia, tubulonephrosis, tubular dilatation, mononuclear cell infiltration in interstinal areas (Fig. 4), and glomerular loop appearance were seen in the kidneys. In the lung, hyperaemia, atelectasia, emphysaema, interstitial thickening, and mononuclear cells in peribronchial, interalveolar interstitium, and perivascular areas were observed. Hyperaemia and haemorrhages in the heart and a decrease in white pulp, an increase in red pulp, and extramedullar haematopoiesis in the spleen were detected.

In case of the TUNEL staining; evident TUNEL positivity in the brain and eyes was seen in group VI. Apoptotic changes were detected in neurons, meningeal cells, and glial cells (Fig. 5) and in the *corpus ciliare*, corneal epithelium (Figs 6-7), anterior lens epithelial cells (Fig. 8) and lens fibers (Fig. 9). In group IV, apoptotic changes were only seen in the eyes. TUNEL positivity of this group was settled in the *corpus ciliare*, corneal epithelium, anterior epithelial lens cells, and lens fibers. In groups I, II, III, and V, apoptosis was lesser than 5% and this staining was not taken into account.

**Table 1**
Histopathologic lesions in the brain and eyes of RFR exposed and control rabbits

| | Control groups | | | RFR groups | | |
|---|---|---|---|---|---|---|
| | I | III | V | II | IV | VI |
| **Brain** | | | | | | |
| Hyperaemia | - | + | - | + | - | - |
| Haemorrhage | - | - | - | + | - | + |
| Neuronal necrobiosis | + | - | + | - | ++ | ++ |
| Clarity of Nissl substance in neurons | - | - | - | - | - | ++ |
| Gliosis | - | + | + | + | + | ++ |
| Mononuclear cells in perivascular areas | - | + | - | - | ++ | - |
| **Eyes** | | | | | | |
| Oedema in cornea | + | - | + | + | ++ | + |
| Mononuclear cells in cornea | - | - | - | + | - | - |
| Epithelial degeneration in cornea | - | - | - | - | - | + |
| Hyperaemia in *corpus ciliare* | - | - | - | + | - | - |

(-) no lesions, (+) mild lesions, (++) moderate lesions, (+++) severe lesions.
I - non pregnant-control, II - non pregnant-RFR exposed, III - pregnant- control, VI – pregnant RFR exposed,
V - newborns from group III, VI - newborns from group IV.

**Table 2**
Histopathologic lesions in the liver, kidneys, lung, heart, and spleen of RFR exposed or control rabbits

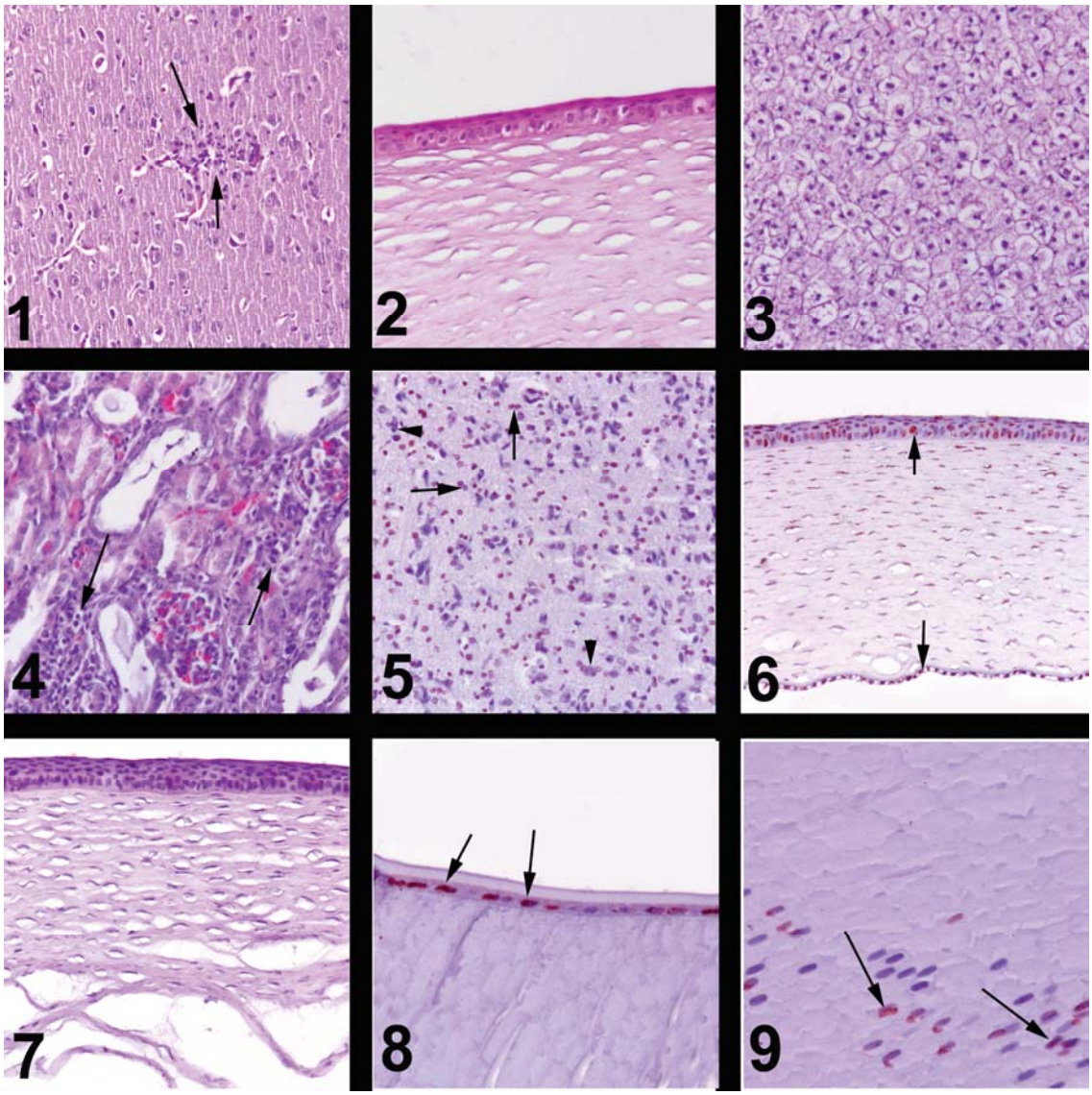
| | Control groups | | | RFR exposed groups | | |
|---|---|---|---|---|---|---|
| | I | III | V | II | IV | VI |
| **Liver** | | | | | | |
| Passive hyperaemia | ++ | ++ | - | + | - | - |
| Degeneration of hepatocytes | + | - | + | +/+++ | +/++ | +/++ |
| Mononuclear cells in sinusoides | - | - | - | - | - | ++ |
| Mononuclear cells in periportal areas | - | - | - | +/++ | ++ | - |
| **Kidneys** | | | | | | |
| Hyperaemia | + | + | + | +++ | +/+++ | - |
| Hydropic degeneration | + | + | - | + | ++ | - |
| Tubular dilatation | - | + | - | ++ | - | - |
| Mononuclear cells in interstitial areas | - | - | - | +/+++ | - | - |
| Glomerular loop appearance | + | - | - | + | + | - |
| **Lung** | | | | | | |
| Hyperaemia | + | - | + | + | - | + |
| Atelectasy | + | - | - | + | - | - |
| Interstitial thickening | - | - | - | + | - | - |
| Emphysoema | - | + | - | + | - | - |
| Mononuclear cells in peribronchial and perivascular areas | + | + | - | +/++ | +++ | - |
| **Heart** | | | | | | |
| Hyperaemia | - | - | + | + | + | + |
| Haemorrhage | - | - | + | + | - | + |
| Hyaline degeneration | + | - | - | +/++ | + | - |
| Myocardosis | + | + | + | ++ | ++ | + |
| **Spleen** | | | | | | |
| Decrease in white pulp, increase in red pulp | ++ | - | - | - | - | - |
| Extramedullar haematopoiesis | - | - | ++ | - | - | - |

(-) no lesions, (+) mild lesions, (++) moderate lesions, (+++) severe lesions
I - non pregnant-control, II - non pregnant-RFR exposed, III - pregnant- control, VI – pregnant RFR exposed,
V - newborns from group III, VI - newborns from group IV.

**Table 3**
TUNEL findings and scores in the brain and eyes of RFR exposed and control rabbits

| | Control groups | | | RFR exposed groups | | |
|---|---|---|---|---|---|---|
| | I | III | V | II | IV | VI |
| **Brain** | | | | | | |
| Neurons | - | - | - | - | - | + |
| Meningeal cells | - | - | - | - | - | +++ |
| Glial cells | - | - | - | - | - | +++ |
| **Eyes** | | | | | | |
| *Corpus ciliare* | - | - | - | - | ++ | +++ |
| Corneal epithelium | - | - | - | - | +++ | +/+++ |
| Anterior epithelial lens cells | - | - | - | - | ++/+++ | ++ |
| Lens fibers | - | - | - | - | +/++ | ++ |

*The intensity of staining was scored as - (0%-5%), + (10%-25%), ++ (26%-50%), and +++ (51% and higher) according to the percentage of positive staining cells.
I - non pregnant-control, II - non pregnant-RFR exposed, III - pregnant- control, VI – pregnant RFR exposed,
V - newborns from group III, VI - newborns from group IV.




**Figs 1-9.** Histopathologic and TUNEL staining results of rabbits RFR exposed or control. 125 x.
1. Gliosis in the brain (arrows), HE. 2. Epithelial degeneration and oedema in cornea, HE. 3. Hydropic degeneration in hepatocytes, HE. 4. Interstitial settled mononuclear cell clusters (arrows) in the kidney, HE. 5. Apoptotic neurons (arrowheads) and glial cells (arrows) in the brain, TUNEL staining. 6. Apoptotic cells in cornea (arrows), TUNEL staining. 7. Control cornea, TUNEL staining. 8. Apoptotic changes in the epithelial cells of the anterior lens (arrows), TUNEL staining. 9. Apoptotic changes in the lens fibers (arrows), TUNEL staining.

## Discussion

The possible relationship between the exposure to RFR emitted by mobile phone radiation and cancer must be correlated with the "non-thermal" effects of the radiation on biological systems due to its insufficient energy to cause significant heating. According to the modelling studies (1, 36), maximum temperature rise calculated in brain tissue is 0.11°C (9). Particularly, the small temperature rise in the brain may be induced by the excitation of the meninx or the first few millimetres of the cortex (32).

However, recent studies have reported that some cellular alterations in cell growth, gene expressions, and in effectors related to cancer development may occur under the exposure of GSM signals (9).

Exposure to GSM signal at 915 MHz can lead to the changes in the gene expression profile by altering the conformation of chromatin in brain cells. However, changes in the gene expression profile were not found to affect the activation and distribution of the chromosomes (3). DNA damages induced by RF fields with its low energy could not be caused directly. It is therefore suggested that the mechanisms acting indirectly could lead to possible DNA damages (34). Up-regulated caspases and gene expressions in neurons and in astrocytes under exposure to GSM signal were determined by Zhao *et al.* (42). The activation of apoptotic effectors induced by cell phone exposure may cause cellular DNA damage (42).

Besides, MW-induced neuronal cell death in the cortex, hippocampus, and basal ganglia was also reported by Salford *et al.* (33) and Ilhan *et al*. (14). In the present study, the formation of apoptotic cells in neurons, meningeal cells, and glial cells was observed after TUNEL staining. Histopathologically, some alterations such as hyperaemia, haemorrhage, neuronal necrobiosis, clarity of Nissl substance, gliosis, and mononuclear cells infiltration in perivascular areas were detected in the brain.

It was reported that radiofrequency energy would lead to ocular effects such as cataract and visual disabilities. Maximum energy absorption and temperature rise can be observed in ocular systems for localised exposure to RF in the range from 0.8 to 10 GHz. It was reported that for the damage of the lens and also potency of the formation of lens opacities, the maximal energy absorption at the threshold levels amounts to 0.918 and 2.45 GHz, respectively (11). One of the main reasons for the formation of lens opacities is the heating induced by the localised RF exposure. The temperature rise in the eye cannot be dissipated effectively because of its encapsulated, avascular, transparent structure, and high percentage of water (8, 41). Therefore, eyeballs can be described as hot spots of radiofrequency radiation (28). Some reports have also demonstrated that changes in the lens and cornea observed through non-thermally RF effect may result from the reductions in ascorbic acid and glutathione levels (2). However, cornea is more susceptible to RF radiation with respect to the lens because of its high polyunsaturated fatty acids content. For this reason, degenerative changes such as oedema, endothelial cell loss, and vacuolisation can be determined in the cornea exposed to localised RFR (19). In the present study, consistent with Kues *et al.* (19) observations, oedema, epithelial degeneration, and mononuclear cells infiltration in the cornea were observed. Moreover, we also observed apoptotic changes in the anterior epithelial cells and fibbers of the lens. In the literature, there is limited number of studies concerning the degenerative effects of RFR on the lens. In this respect, our results would offer new data.

Numerous studies have demonstrated that degenerative changes of different tissues induced by mobile phones radiation have been based on oxidative stress (2). In the oxidative stress, reactive oxygen species (ROS) generated by external field application may interact with cellular biomolecules, such as lipids, proteins, and DNA, leading to modification and damage (6). Ilhan *et al.* (14) determined that mobile phone exposure may induce oxidative stress mediated by ROS in brain tissues of rats. Ozguner *et al*. (31) observed the oxidative stress induced impairment in myocardial tissue under mobile phone exposure. Authors found that this impairment could return to normal conditions by the administration of external antioxidant. Koyu *et al.* (18) reported that exposure to 1,800 MHz microwave (MW) would be effective in the induction of oxidative stress *via* increased free radicals in liver tissues. Moreover, Oktem *et al.* (29) demonstrated that exposure to mobile phone irradiation had a significant effects on kidney tissues by activating ROS. Furthermore, Meral *et al.* (27) revealed that RFR generated from cellular phone may produce oxidative stress by increasing lipid peroxidation in brain tissues. On the contrary, Irmak *et al.* (15) reported that mobile phone radiation did not change the lipid peroxidation level.

Oxidative stress induced by RFR may arise from the stimulation of different amounts of polyunsaturated fatty acids and protein compositions of tissues. It is suggested that both polyunsaturated fatty acids and protein synthesis may be essential for initiation of apoptosis under external stimuli (2, 24). In the present study, histopathological changes under 1,800 MHz RFR were observed in the liver, kidneys, lung, heart, and spleen. Hyperaemia induced by RFR was found commonly in the liver, kidneys, lung, and heart. Mononuclear cell infiltration was also observed widely in the liver, kidneys, and lung. Degeneration in hepatocytes and tubular epithelium, tubular dilatation, atelectasiae, emphysoema, interalveolar interstitial thickening in the lung, haemorrhage, myocardosis in the heart, extramedullar haematopoiesis in splenic tissues were the other changes resulted by RFR exposure.

Consequently, our results support the suggestion that exposure to 1,800 MHz may induce some pathomorphological alterations in different tissues of non-pregnant and pregnant rabbits and their infants. Studies on the effects of mobile phone radiation on pregnant animals and their offspring conducted with the use of histopathological and immunohistochemical

methods are absent in the literature. With this perspective, our results may constitute a reference for the future research concerning pregnancy. Moreover, it would be significant to increase the number of these studies for establishing international standards for the protection of pregnant women under RFR exposure.

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