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The Effects of Exposure to 900 MHz Radiofrequency Radiation and Nicotine on Apoptotic Ratio of Human Fetal Cells

Mustafa Emre[1], Ayper Boğa[2]*, Salih Cetiner[3], Erdal Tunc[4], Osman Demırhan[4]

[1]Department of Biophysics, Faculty of Medical, Cukurova University, Adana, Turkey; [2]Department of Physiology, Faculty of Medicine, Cukurova University, Adana, Turkey; [3]Central Laboratory, Faculty of Medicine, Cukurova University, Adana, Turkey; [4]Department of Medical Biology and Genetics, Faculty of Medicine, Cukurova University, Adana, Turkey

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***Correspondence:** Ayper Boğa, Department of Physiology, Faculty of Medical, Cukurova University, 01330 Balcalı, Adana, Turkey. E-mail: aypbog@cu.edu.tr
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Funding: The results showed that exposure of fetuses to 900 MHz RF-EMR may not be as harmful as previously assumed. Findings also suggest that combined exposure to tobacco and cell phone radiation may lead to more damaging effects on fetuses.
Competing Interest: The authors have declared that no competing interest exists

Abstract

AIM: The present study purposes to examine the effects of radiofrequency electromagnetic radiation (RF-EMR) and nicotine exposures on apoptosis and necrosis ratio of cultured amniocytes.

METHODS: Amniotic fluid samples were gained from 48 pregnant women and, after culturing procedure, samples were analyzed by flow cytometry.

RESULTS: Significant increases were observed in ratios of necrotic (UL), early (LR), and late apoptotic cells (UR) in the exposure groups of RF-EMR, nicotine, and RF-EMR + nicotine groups ($p < 0.01$ and $p < 0.001$), along with significant changes in ratios of viable (LL) cells ($p < 0.001$).

CONCLUSION: Our results showed that nicotine exposure, alone or in combination with 900 MHz RF-EMR, led to increased apoptotic cell ratios. Amniocytes were even more severely affected by nicotine, when nicotine was administered in combination with 900 MHz RF-EMR, due to created synergistic effect. More studies with different settings, exposure times, frequencies, and doses are needed to clarify net effects of exposures to nicotine and RF-EMR radiation, alone or in combination.

Introduction

Apoptosis, as a form of programmed cell death, is featured by cell shrinkage, fragmentation, and disposal with no sign of inflammation. Apoptosis is categorized according to causing factors which trigger apoptotic process. Intrinsic apoptosis which is seen during the development of tissues and aging is generally stimulated by internal factors. Internal factors leading to apoptosis include DNA damage, endoplasmic reticulum stress, reactive oxygen species overload, replication stress, microtubular alterations, or mitotic defects. Sometimes, external factors such as radiation might lead to apoptosis which is called extrinsic apoptosis. The extrinsic signals induce transmembrane receptor-mediated interactions and this interactions triggers pathways, leading to apoptosis. Tumor necrosis factor receptor gene superfamily constitutes an important fraction of these receptors types. In contrast to apoptosis, necrosis has traditionally been considered to be an unregulated form of cell death which typically occurs after traumas. The hallmarks of necrosis are dysfunctional cell membrane, cell swelling, and inflammation [1-3].

In the past decades, the use of cell phones has become increasingly more common and widespread across the world, to the extent that they now represent an indispensable requirement of daily life in the 21st century. However, the common use of cell phones and similar devices has also raised concern regarding the potential health hazards that might result from exposure to radiation emanating from these devices. There are also growing concerns regarding the long-term effects associated with continuous exposure to radiofrequency electromagnetic radiation (RF-EMR) from such devices [4,5]. To date, many studies investigating the consequences of cell phone radiofrequency radiation on human health have been conducted. Studies conducted on adults gave contradictory results, therefore, more recent studies have particularly focused on the consequences of radiation types on vulnerable groups, such as pregnant women, unborn children, and the elderly [6]. The International Commission on Non-Ionizing Radiation Protection [7] and the Institute of Electrical and Electronics Engineers [8] determined exposure limits for public environments and workplaces. However, those institutions did not specify any exposure limits for the above-mentioned

vulnerable groups [6]. Specific absorption rate (SAR) specifies the rate of RF-EMR absorbed by human tissues. This rate was determined by the European Union and the United States as 2.0 W/kg and 1.6 W/kg, respectively. However, today, we still do not know whether exposure to RF-EMR within SAR limits has any effect on fetuses [9].

More than 60 years ago, the first important data regarding the carcinogenic effects of tobacco smoke have been reported [10]. Nicotine is an important compound found in tobacco smoke and appears to play a significant role in the etiology of many diseases and disorders [11]. Nicotine also causes DNA damage which might trigger apoptosis [12]. Some researchers have proposed that nicotine shows apoptosis causing effects on the fetal cells, possibly by increasing oxidative stress in relevant fetal sites [13]. Many studies also showed that tobacco smoke and its biological byproducts can cause DNA damage and are directly involved in the triggering of cancers [14,15]. Despite cigarette smoking is a leading cause of numerous harmful obstetrical and fetal consequences, approximately 15–20% of all women maintain their smoking habit during pregnancy. More than 4800 different compounds are found in smoke of tobacco and approximately 60 of these compounds are carcinogenic to animals and humans (Group 1) (IARC) [16]. Nicotine exposure during intrauterine life might lead to long-term persistent results on extrauterine life [17]. Nicotine which is also accepted as a drug shows significant cultural, economic, and health impacts and implications on society [18-21]. In this study, we aimed to assay the effects of RF-EMR and nicotine exposures on apoptosis and necrosis ratio of cultured amniocytes.

Materials and Methods

Sample obtaining

Amniotic fluid samples were collected from pregnant women with gestational ages of more than 14 week, for examining the consequences of RF-EMR exposure and nicotine sulfate (Acros Organics, product 41565, cas no: 65-30-5, New Jersey) administration on amniocytes.

Those women were applied to Gynecology and Obstetrics Department of Balcali Research and Training Hospital in Cukurova University for routine pregnancy examination and prenatal chromosome analysis. Five milliliters of amniotic fluid samples were taken from patients, 48 pregnant women, under sterile conditions and those samples were rapidly transferred to our laboratory, culture laboratory of Medical Biology and Genetics Department of Çukurova University. The experimental protocols were approved by the local non-interventional clinical research ethics committee of the Cukurova University Medical Sciences (Meeting/decision number: 030/21). In the implementation of the procedures of this study, NIH guidelines for research using human specimens, cell lines, or data were followed.

RF-EMR exposure system and design

In this study, the cultured fetal cells were irradiated using GSM signal generator obtained from set Electronic, Co. Ltd. (Model GHZ2011X, Sakarya, Turkey). A radiofrequency of 900 MHz (equivalent to signal produced by any digital mobile phone for personal usage) was applied using frequency-modulated continuous waves (wave impedance = E (Electric field strength)/H(magnetic field strength) = Z (Wave Impedance); 373 Ω). 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 the GSM.

Reflection and exposure levels determining measurements were performed using a standard probe of RF measurement system (portable RF meter, TES-593, Electrosmog Meter, Taiwan). The probe measured the values indicating reflection and exposure levels throughout X, Y, and Z axes, added these vectorial values, and provided end results. In the area of the samples, electric and magnetic field strengths were measured as 26 V/m ± 9% and 0.067 A/m, respectively. The average power density (ExH) was measured using a calibrated detector and radiofrequency EMF meter and the value was recorded as 1.74 W/m^2. The distance between antenna and culture flasks was kept at 1 cm, which is accepted as the possible shortest distance in such a setting. The electromagnetic dose and the SAR were calculated according to electric field density (V/m) and power density (V/m^2) measurements. SAR values, which were calculated according to Dabrowski *et al*. – used method, ranged between 0.025 and 0.034 W/kg [22]. Consequently, under the same antenna's power and orientation conditions, average SAR value was calculated as 0.033 W/kg. The study was conducted in a chamber which can easily shield RF radiation of as high as 900 MHz and 100 dB. The cell culture flasks placed in the anechoic chamber (a cube of 20 × 20 × 20) were exposed to RF-EMR (900 MHz, 26 V/m, SAR 0.033 W/kg) for 6 h per day for 8 days. Control cultures were not exposed to RF-EMR (Figure 1).

Culturing procedure and flow cytometric examination

Amniotic fluid samples in sterile syringes were transferred into 15 mL sterile tubes inside laminar flow device (Kojair) under sterile conditions. Each sample was equally divided between four tubes, each tube

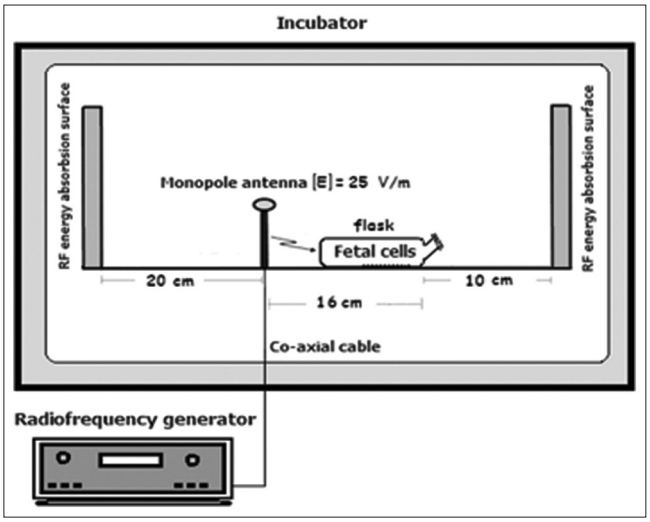

Figure 1: During the study, a GSM signal generator was used to generate radiofrequency electromagnetic radiation (RF-EMR). The RF field was set to 25 V/m. The RF-EMR exposure system applied 900 MHz RF to fetal cells

containing 1.25 mL fluid; thereby, four group of samples, a control group, and three study groups (RF-EMR, nicotine, and RF-EMR + nicotine exposed groups) were constituted. After centrifugation at 800 rpm for 5 min, supernatants were depleted from tubes and pellets were poured into culture flasks, containing 2 mL culturing medium, Amniochrome Plus Media (Lonza). Standard amniocyte culturing method was followed for all samples. RF-EMR group samples were cultured in the set-up described in Figure 1 without addition of nicotine sulfate. For nicotine and RF-EMR+nicotine exposed groups, at the beginning of the culturing procedure, nicotine sulfate was added to the culture flasks at the dose of 25 ng/mL (in fetal serum – range 0.5–25 ng/mL) [23]. The culture flasks were then incubated at 37°C and under 5% CO_2 and adequate humidity. At the 8th day of the culturing time, the culture flasks were controlled under inverted microscope, and the growth conditions of all flasks were recorded. Attached cells to flask substratum and grown cell foci were observed in all flasks. For changing flask media, old media were poured into a glass container, and fresh media were added to the flasks (2 mL for each flask) inside a laminar flow device and under sterile conditions; in the case of nicotine and RF-EMR+nicotine exposed groups, this was then followed by the addition of nicotine sulfate to the flasks at the doses of 25 ng/mL. During the 8-day culture period, samples in the RF-EMR and RF-EMR +nicotine groups were exposed to 900 MHz RF-EMR for 6 h per day. At the end of the culturing period, flasks were checked again and adequate cell growth was observed and then harvesting process was initiated.

First, colchicine (4.5 mL) was applied to each flask for 4 h; then, cell colonies that had attached to the base of the flasks were detached and suspended in Trypsin + EDTA solutions. The suspended cells were then centrifuged twice at 800 rpm for 5 min (with PBS solution being used to wash the cells) and transferred to sterile tubes. The resulting cell suspensions were diluted and adjusted with the Annexin-V binding solution (Annexin V apoptosis assay) to obtain a cell density of 2–3 × 10^6 cell/mL. Following this, a 100 μL aliquot was taken from each tube, and 5 μL Annexin V – fluorescein isothiocyanate and 10 μL propidium iodide were added to each 100 μL aliquot. Finally, cell counts of samples were determined by flow cytometry.

Statistical analysis

The Statistical Package for the Social Sciences 11.5 package program was used for statistical analysis. For analyzing the study results, one-way analysis of variance and *post hoc* Tukey HSD for multiple comparison tests were used. $p < 0.05$ was accepted as indicative of statistically significant difference between groups.

Results

Percentages of viable (LL), necrotic (UL), early apoptotic (LR), and late apoptotic (UR) fetal cells

Amniocytes (cultured fetal cells) in the study groups were exposed to RF-EMR, nicotine, or a combination of RF-EMR and nicotine. The distributions and percentages of viable (LL), early apoptotic (LR), late apoptotic (UR), and necrotic (UL) fetal cells in the control, 900 MHz RF-EMR, nicotine, and 900 MHz RF-EMR + nicotine exposure groups are presented in Figures 2 and 3. Significant effects were observed in the RF-EMR, nicotine, and RF-EMR + nicotine groups as a result of exposure. The ratios of viable cell (LL)

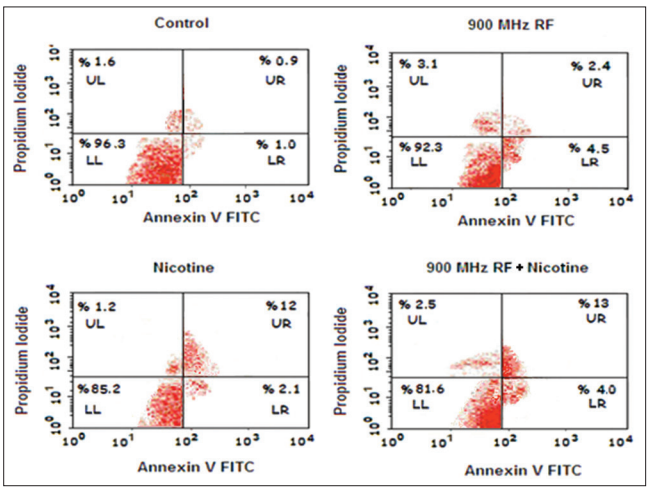

Figure 2: Evaluation by flow cytometric analysis of the distribution of viable (LL), early apoptotic (LR), late apoptotic (UR), and necrotic (UL) cells between the 900 MHz radiofrequency electromagnetic radiation (RF-EMR), nicotine, and 900 MHz RF-EMR + nicotine exposure groups

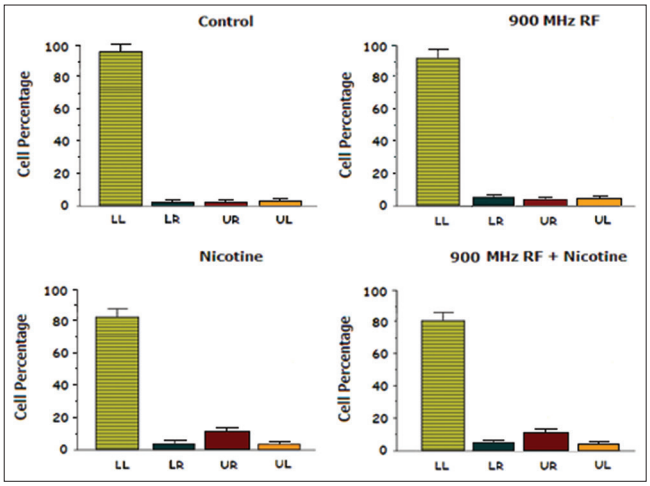

Figure 3: The percentage of viable (LL), early apoptotic (LR), late apoptotic (UR), and necrotic (UL) fetal cells in the 900 MHz RF-EMR, nicotine, and 900 MHz RF-EMR + nicotine exposure groups

and interactions in all aforementioned groups were F (3.36 = 63,943 [p < 0.001]). The percentages of viable cells of control, 900 MHz RF-EMR, nicotine, and 900 MHz RF-EMR + nicotine exposure groups were 96.2% ± 0.46, 92.2% ± 0.9, 85.1% ± 0.97, and 81.4% ± 1.01, respectively.

The percentages of viable cells in the study groups were significantly different from the percentage of viable cells in the control group ([p < 0.001] [except RF group, p = 0.008]). The percentage of necrotic cells (UL) and all group interactions was F (3.36 = 68.670 [p < 0.001]). Significant difference (p < 0.001) was observed between the percentages of necrotic cells in the 900 MHz RF-EMR exposure (3.1 ± 0.09) and control groups (1.6% ± 0.07). In the nicotine and the 900 MHz RF-EMR + nicotine exposure groups, the percentages of necrotic cells were 1.3% ± 0.09 and 2.6 ± 0.136, respectively, showing significant difference from control group (p < 0.001). The percentage of early apoptosis (LR) and all group interactions was F (3.36 = 133,549 p < 0.001). Significant difference (p <0.01) was observed between the percentages of early apoptotic cells in the 900 MHz RF-EMR exposure (4.5% ± 0.19) and control groups (1.01 ± 0.07). In the nicotine and the 900 MHz RF-EMR + nicotine exposure groups, the percentages of early apoptotic cells were 2.1% ± 0.117 and 4.0% ± 0.148, respectively. There is also significant difference between control and those groups (p < 0.001).

The percentage of late apoptosis (UR) and all groups interactions was F (3.36 = 204,357 [p < 0.001]) (except RF, p = 0.095). The percentages of late apoptotic cells of control, 900 MHz RF-EMR, nicotine, and 900 MHz RF-EMR + nicotine exposure groups were 0.9% ± 0.07, 2.47 ± 0.15, 12% ± 0.52, and 13% ± 0.71, respectively. The percentages of late apoptotic cells in the study groups were significantly different from the percentage of late apoptotic cells in the control group (p < 0.01) (Figures 2 and 3).

Discussion

In all groups of the present study, the percentages of viable, necrotic, and early/late phase apoptotic cells were determined using flow cytometric methods. Annexin V is a marker that tags externalized phosphatidylserine on the membranes of cultured cells.

Since phosphatidylserine externalization occurs during the initiation phase of apoptosis, the marking of this acidic phospholipid rendered possible visualization of apoptotic cells under fluorescence microscopy [24]. After initiation of apoptosis; damaged cells (e.g., cells subject to toxin- or irradiation-related damage) will exhibit certain chemical changes and processes, free radical generation within mitochondria, and the activation of caspases. Free radical generation by mitochondria, in particular, causes oxidative damage to nuclear DNA and other cellular structures, which, in turn, triggers cell death through the extensive breakdown of nuclear DNA [24]. In combination with Annexin V, propidium iodide (Annexin V/propidium iodide system) enables researchers to distinguish viable, early apoptotic, late apoptotic, and necrotic cells through flow cytometry [25]. In the present study, flow cytometry and Annexin V/propidium iodide system were used to detect viable, early apoptotic, late apoptotic, and necrotic cells.

The potential health risks presented by EMR, in particular by GSM-like RF-EMR, are a subject of considerable interest and concern both among the public and the scientific community [26]. To date, a large number of studies have been managed regarding possible harmful effects (toxic, mutagenic, or carcinogenic effects) of RF-EMR. In our study, viable cells ratios of study groups were significantly different from viable cells ratio of control group. Similarly, in a study conducted on bronchial epithelial cell of mice, it was reported that exposure to nicotine at concentrations of 1.25 mM (approximately 200 ng/ml), 2.5 mM, and 5 mM decreased cell viability to approximately 70%, 50%, and 20%, respectively; so, study results showed that nicotine exposure decreased cell viability in a dose-dependent manner [27]. In another study conducted on MG-63 osteoblast-like cells, it was reported that cell viability decreased to beneath 60% (cell viability, % of control) on exposure to 5 mM nicotine with the duration of 72 h [28].

In comparison to our results, those studies reported more dramatically decreased viable cell proportions on exposure to more concentrated doses of nicotine. In a study investigated the effect of 900 MHz RF radiations on the human hepatocellular carcinoma cell line, Hep G2 cells, for an exposure periods of 1, 2, 3, and 4 h, it was reported that significant decreases were observed in ratios of viable cells in study samples of 3 and 4 h exposure times (48% and 41%, respectively) compared to controls (57%) (p < 0.05) [29]. In another

study, the effects of 960 MHz microwave radiations on human epithelial amnion cells were examined. After 40 min exposure, it was reported that cell proliferation ratio was significantly decreased, a decrease of 10.3% at SAR of 2.1 mW kg^{-1} ($p < 0.05$) [30].

It is a well-known phenomenon that radiation has an effect of inducing necrosis. This effect of radiation is used in radiotherapy with the aim of eliminating harmful neoplastic cells in targeted tissues. Therefore, radiotherapy, particularly gamma radiation therapy, plays a significant role in the treatment of tumors. In a review paper focused on primary and metastatic brain tumors, it was reported that radiation-induced necrosis could vary between 4.1 and 30%; depending on duration, applied dose, and chosen technique of treatment [31]. In a study of low MHz frequency but long time intermittent exposure, Sprague-Dawley rats were subjected to 20 MHz continuous wave radiofrequency radiation (SAR of 0.3 W/kg) for 6 h/day, 5 days/week up to 6 weeks. After initiation of exposure, on days 8, 22, 39, and 42, randomly selected rats were killed. Those rats showed no statistically significant differences from control rats in terms of body mass, spleen cell density, erythrocyte and leukocyte counts, hematocrit, and erythrocyte fragility [32]. A similar study was conducted on 10-week-old hairless female rats (IFFA CREDO, L'Arbresle, France). In this study, female rats were exposed to 900 or –1800 MHz RFR (SAR was 5 W/kg) for 2 h/day over 2 weeks and effects of those doses of radiation on necrosis and proliferation rates on skin cells were examined.

Results showed that those doses of radiation did not affect necrosis and proliferation rates of epidermal cells [33]. In our study, necrotic cells ratios in study groups were significantly different from the control group ($p < 0.001$).

There was a significant difference between ratios of early and late apoptotic cells of the study and control groups. In a study, conducted on Wistar rats, the effect of RF- and microwave (MW-) EMR on bone marrow cells was investigated. In this study, 2.45 GHz of either RF- or MW-EMR was applied to a subgroup of rats for 2 h per day of a week, while rats in four other subgroups were continuously exposed to the same level of radiation for 4, 16, 30, and 60 h. Results of this study showed that RF/MW irradiation led to an increase in the formation of micronucleated immature red cells in the bone marrow [34]. In another study, utilizing cell viability test, the effects of high-frequency EMR on cell proliferation rate and apoptosis induction were evaluated [35]. At short (2–12 h) and long (24–48 h) exposure times, the results indicated that subjection to 900 MHz continuous waves could induce a quick self-defense response stimulated by DNA damage and confer to the survivor cultured cells a further advantage for survival and proliferation. In another study, it was demonstrated that EMR exposure may affect cellular growth and proliferation and the induction of apoptosis [36]. In a similar study, it was suggested that RF- and MW-EMR of high frequency and intensity might indirectly cause damage at the level of DNA by interfering with gene replication and transcription processes [37]. Similarly, many studies that have been conducted to date have demonstrated that certain frequencies of RF-EMR may trigger various responses at a biological level, including apoptosis [35, 38, 39]. In our study, we observed that exposure to RF-EMR (e.g., cell phones radiofrequency) is partly harmful to human fetal cells. The ratio of necrotic and apoptotic fetal cells in the RF-EMR exposure group was significantly lower in comparison to the nicotine and RF-EMR + nicotine exposure groups ($p < 0.001$).

Hence, our results are in accordance with results of many studies [34-36, 38, 39], but are in contradiction with the results of some other studies [4, 40, 41].

In rodents, tobacco smoke is known to trigger alterations in chromosome structure and the formation of micronuclei in cells, particularly in lung and bone marrow cells, a hallmark indicator of apoptosis. A recent study examined the effects of nicotine sulfate on proliferating fetal cells in media that contained nicotine at a concentration of 25 ng/ml [42]. The results of this study demonstrated that nicotine caused strong genotoxicity on human fetal cells, leading to suggestion that there might be a link between nicotine intake through smoke and aneuploidy formation during pregnancy [42]. In our study, 25 ng/mL nicotine doses were also administered to human amniocytes to observe its genotoxic effects. We also demonstrated that subjection to nicotine alone resulted in far more apoptotic and necrotic cells than exposure to 900 MHz RF-EMR.

Conclusion

There are considerable differences in the outcomes and conclusions reached by many studies that have been conducted until now to assess the apoptotic and genotoxic effects of RF-EMR. However, certain results and observations appear to be consistent between different studies [4, 34-36, 38-41, 43]. Our opinion is that low frequencies of RF-EMR such as GSM-like (900 MHz) RF-EMR may have deregulative and partly harmful effects on apoptosis stimulation. Amniocytes and fetal cells may be a more vulnerable cell group in terms of damaging effects of RF-EMR. Many study results indicated that nicotine has damaging effects on apoptotic pathways and ultimately can lead to anti-apoptotic or pro-apoptotic effects [21, 44, 45]. Our results showed that nicotine exposure, alone or in combination with 900 MHz RF-EMR, led to increased apoptotic cell ratios. Amniocytes were even more severely affected by nicotine, when nicotine was administered in combination with 900 MHz RF-EMR,

due to created synergistic effect. More studies with different settings, exposure times, frequencies, and doses are needed to clarify net effects of exposures to nicotine and RF-EMR radiation, alone or in combination.

Acknowledgments

This work was approved by Çukurova University Faculty of Medicine Non-Invasive Clinical Studies Ethics Committee, Decision number 30(21), at April 4, 2014.

Authors' Contributions

Mustafa Emre and Salih Cetiner conceived, designed and performed the study. Ayper Boğa Pekmezekmek performed the experiments and wrote the paper. Erdal Tunc wrote the paper. Erdal Tunc – revisioned the paper.

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