CLINICAL STUDY

Assessment of the effects of radiofrequency radiation on human colon epithelium cells

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ABSTRACT
OBJECTIVES: The aim of the study was to investigate the possible effects of radiofrequency radiation (RFR) at different frequencies for different exposure durations on caspase-dependent apoptosis pathways in human colon adenocarcinoma (HT-29).
METHODS: HT-29 cells were exposed to 1800 MHz; 2100 MHz and 2600 MHz RFR for 3 h cont., 6 h int. and 6 h cont.. Cell viability measurements were performed by Trypan Blue exclusion assay and the gene expressions of CASP8, CASP9, CASP3 and CASP12 were analyzed using qRT-PCR.
RESULTS: Exposure to 2100 MHz RFR for all 3 durations of exposures was more effective for the ratio of the number of viable HT-29 cells w.r.t 1800 MHz RFR and 2600 MHz RFR exposures. After 2100 MHz RFR exposure, caspase activation increased significantly (for 3h cont. and 6 h int. exposures CASP8 and CASP9 levels; for 6 h cont. exposure CASP3 levels) (p < 0.05). Exposures to both 1800 MHz and 2600 MHz RFR for 3 different exposure durations did not change the activation of caspases we analyzed in this study (p > 0.05). CONCLUSION: Decreases in the cell viability of HT-29 cells for certain frequencies and also durations are consistent with significant increases in caspase activations. The results of caspase activation after 1800 MHz or 2600 MHz RFR exposures can be interpreted as the activation of different types of cell death pathway by caspase signaling cascades (Fig. 15, Ref. 56). Text in PDF www.elis.sk.
KEY WORDS: radiofrequency field, HT-29, apoptosis, caspase activation, in vitro.

Introduction

All living organisms, including human beings, live in harmony with the natural electric (E) and magnetic (B) fields of the Earth because of the highly specialized and sensitive structure of their cells/ tissue against Electromagnetic (EM) fields. The sensitive and dynamic balance between living organisms and natural EM fields has been disrupted by the rapid development of artificial (man-made) EM technologies in the frequency ranges of 0 Hz to 300 GHz [mobile phones, base stations, microwave ovens, radars, power lines, TV and radio transmitters, computers, electrical appliances etc.] (Panagopoulos, 2013). Intensive exposure to EM fields including Radiofrequency (RF) and Extremely Low Frequency (ELF) fields, in our everyday lives has been accepted as new types of environmental pollution (Redlarski et al, 2015). Meanwhile, the incidence and mortality rates of various cancer types have increased due to the influence of negative environmental conditions and as a consequence of abnormalities in genetic material (Ahlbom and Feychting, 2003).

The underlying mechanisms of biological responses associated with RF radiation exposure can be explained by two different approaches: thermal and non-thermal.

Interaction of RF fields with living systems has been characterized by its frequency, intensity, and exposure duration. However, interaction mechanisms are mainly based on the absorption of RF energy by biological matter. In the thermal mechanism, the RF electric field generates an oscillating current and the rapid transfer of the energy of this current into the molecular motion responsible for most of the heat capacity results in an increase in the local temperature. In contrast to thermal mechanism, non-thermal mechanism are based on the physical or biochemical changes in target cell, tissue, or organism but not related to temperature increase or tissue heating (Belyaev, 2005a, b). Moreover, Fröhlich (1968) has suggested that RF/MW range could create a resonance in the biomolecules. According to this suggestion, incident RF energy may be captured by a large group of oscillating dipoles and integrated into a single mode of coherent vibrational energy.

In the interaction mechanism of electromagnetic fields with biological system, it is noteworthy that not attenuated EM fields passed through cells can interact directly with the nuclear DNA and other structural molecules. With the induction of electric oscillations; the disturbance of cell membrane proteins, activation of enzyme cascades which transfer cell surface signals to the intracellular system, including cell nuclei and cell growth and division appear (IARC Monographs on the Evaluation of Carcinogenic...
Risks to Humans, IARC, 2013).

Cell cycle controls by tumor suppressor genes, DNA damage and repair mechanism, and apoptosis have played a critical role during the carcinogenesis process. However, induced resistance to programmed cell death is considered as a common characteristic of cancer cells. When an error occurs in the stages of chromosomal arrangement, DNA repair or DNA replication, cell cycle stops until all errors are repaired. Nevertheless, if there are irreversible errors or damages, apoptosis pathways are activated. And thus, the maintenance of balanced and healthy internal conditions (homeostatic balance) is ensured. Apoptosis, a vital biological process for several physiological and pathological events, enables the treatment of various diseases such as cancer, AIDS, autoimmune disorders etc. using biochemical methods and genetic components that either activate or inhibit. (Tomatır, 2003; Alberts et al, 1994).

Therefore, to examine whether the impact of radiofrequency (RF) fields on cancer occurrence or treatment, the assessment of probable effects of EM fields on apoptosis mechanisms should be one of the most effective approaches.

It is important to understand and clarify that EM fields emitted from advanced technological devices that make our lives easier have contributed to an increase of the incidence of cancer as environmental risk factors and pollutants. Therefore, scientists have been working intensely on the probable link between EM fields-cancer interactions for over thirty-five years. According to literature, scientists and researchers began to develop an interest in two subjects: 1. there is a close relationship between EM fields and cancer incidence and 2. It is proposed that EM Fields can be used in cancer treatment as an adjuvant therapy.

International Agency for Research on Cancer [IARC-part of World Health Organization (WHO)] coordinated a feasibility study in 1998 and 1999, which concluded that an international study of the relationship between mobile phone use and brain tumor risk would be feasible and informative. The WHO/International Agency for Research on Cancer (IARC) has classified radiofrequency electromagnetic fields as possibly carcinogenic to humans (Group 2B), based on an increased risk for glioma, a malignant type of brain cancer, associated with wireless phone use (THE WHO/IARC PRESS RELEASE NO 208, 31 May 2011).

In the present study, the probable effects of wireless technologies at different frequencies [GSM-like signal (1800 MHz), 3G UMTS mobile communications (2100 MHz) and 4G Long Term Evolution (LTE) (2600 MHz)] on caspases-dependent apoptosis mechanisms in human colorectal adenocarcinoma cells were investigated for different exposure durations [3h continuous, 6h intermittent and 6h continuous].

Materials and methods

Cell culture

Human colorectal adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC; USA). HT-29 (ATCC®HTB-38) was cultured in Dulbecco’s modified eagle’s medium (Biochrom-Merck GmbH, Germany) supplemented with 10 % Foetal bovine Serum (FBS, F4135, USA origin Heat Inactivated, sterile-filtered, suitable for cell culture, Sigma-Aldrich-Merck, GmbH, Germany) and 1 % penicillin/streptomycin (Solution stabilized with 10,000 units penicillin and 10 mg streptomycin/mL, sterile-filtered suitable for cell culture, Sigma-Aldrich-Merck, GmbH, Germany). Cells were grown to confluence in T75 flasks (Nest Biotecchnology, USA) in a 37 ºC incubator (Nüve, EN120, Ankara, Turkey) at 5 % CO2. HT-29 cells were passaged after growing to 90 % confluency and replaced equally (1x106cells/ml) into the sterile 35 cm² Petri dishes for RF radiation exposure.

RF radiation exposure system

The RF exposure system mainly consists of

- a plexiglass box (32x52x75 cm) covered by RF absorber foam (1–17 GHz, 5 mm, 3500 Microwave absorber foam, Holland Shielding Systems BV, The Netherlands) for the elimination of both external and internal reflections,
- a vector signal generator (Rohde &Schwarz SMBV 100A, 9 kHz–3.2 GHz, München, Germany) for the generation of digitally modulated RF signals.
- a horn antenna (Schwarzbeck, Doppelsteg Breitband Horn antenna BBHA 9120 L3F, 0.5–2.8 GHz, Schöna, Germany) for the emissions of the RF signals generated by the vector signal generator.
- a digital microscope hot plate unit (Nikon,Thermo Plate, Japan) for keeping the stability of cells’ temperatures.

The output power and the frequency were controlled by a spectrum analyzer (Rohde & Schwarz, FSH 18, 10 MHz–18 GHz, München, Germany) integrated to the signal generator. The distance between the horn antenna and the cell culture plates was 20 cm to provide far field conditions. Environmental Radiofrequency Electric (E) and Magnetic (B) Fields were measured by using NARDA EMR-300 device and its E field probe (100 kHz–3 GHz) and NARDA EMF 300 and its B field probe (5 Hz–32 kHz) (Narda, Pfullingen, Germany) (Fig. 1).

Human colorectal cells were exposed to 1800 MHz, 2100 MHz and 2600 MHz RF radiations with a SAR of 1.6 W/kg for: i. 3 hours continuously, ii. 6 hours intermittently [3 hours RF exposure setup on/1 hour RF exposure setup off (At this time, cells were kept in a 5 % CO2 incubator)/3 hours RF exposure setup on], iii. 6 hours continuously in order to investigate the probable effects of RF radiation on caspase-dependent programmed cell death. The incident power generated by the signal generator was 1 W, and the measured output E field values were 31.20 ± 0.95 V/m for 1800 MHz, 31.56 ± 0.27 V/m for 2100 MHz and 31.43 ± 1.15 V/m for 2600 MHz. The direction of the RF propagation was perpendicular to the diameters of the wells.

Before the analysis of gene expression, the percentage of cell viability between the control, sham and RF radiation exposure groups were determined by trypan blue staining assay. These experiments were repeated at least three times.

Semi-quantitative RT-PCR

Total RNA was extracted from HT-29 cell line with Trizol reagent according to the manufacturer’s protocol (Roche Diagnostics GmbH, Mannheim, Germany). The concentration of the extract-
ed total RNA was assessed by using NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Its purity and integrity were determined by ratio of absorbance at 240/260 and 280/260 nanometers. The measured RNA purities of whole samples are in the range of 1.8–2.0. One microgram of total RNA was reverse transcribed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). PCR reactions were in a 20 μl total volume according to manufacturer’s protocol using Template-Primer Mix, Master Mix, 5μl cDNA and the primers for a) CASP8 5’ TTTCTGCCTACAGGGTATGC 3’ (Forward) and 5’GTCCAACTTTCTCTTCCTCA3’ (Reverse) b) CASP9 5’CTGTCTACGCGCAGATGGAT3’ (Forward) and 5’GGGACTCGTTCAAGGGAA3’ (Reverse) c) CASP3 5’CATGGAAGCGAATCAATGGACT3’ (Forward) and 5’CTGTACCAGACCGAGATGTCA 3’ (Reverse) d) CASP12 5’AA CAACCGTAACTGCCCAGAGT3’ (Forward) and 5’ CTGCACGGGTTTTCCACT3’ (Reverse) e) Housekeeping control gene 5’GGCACCCAGCACAATGAAG3’ (Forward) and 5’CCGATCCACGAGTACTTG3’ (Reverse). PCR reactions were briefly as 95 °C for 5 min followed by 40 cycles at 95 °C for 10 sec, 50 °C for 20 sec and 72 °C for 30 sec, 1 cycle of 72 °C for 7 min and extension at 4 °C. The relative expression level of the genes was calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001).

Results

The results of comparing cell viability are shown in Figure 2 a, b, c.

1800 MHz RF radiation exposure

Cell viability

We observed the percentage rates of cell viabilities of HT-29 cells exposed to 1800 MHz GSM-like RF radiation for three hours (continuous) and six hours (intermittent) [3 hours RF exposure setup on/1 hour RF exposure setup off/3 hours RF exposure setup on] and they did not change significantly with respect to their negative control and sham groups (p > 0.05). However, a significant decrement in six hours (continuous) exposure groups was determined (p < 0.05).

Genetic analysis

We analyzed the gene expression levels of CASP8, CASP9, CASP3 and CASP12, they did not change significantly with the exposure to 1800 MHz GSM-like RF radiation for all three exposure durations compared to their negative control and sham groups (p > 0.05) (Figs 3–6).

2100 MHz RF radiation exposure

Cell viability

Significantly decrements were observed in the percentage rates of cell viabilities of HT-29 cells exposed to 2100 MHz GSM-like signals for all three exposure durations compared to their negative controls and sham groups (p < 0.05).
Genetic analysis

We found significant increases in the gene expression levels of CASP8 and CASP9 of HT-29 cells exposed to 2100 MHz GSM-like signals for three hours (continuous) and six hours (intermittent) [3 hours on/1 hour off/3 hours on] with respect to their negative control and sham groups (p < 0.05).

The expression levels of CASP3 of HT-29 cells exposed to 2100 MHz RF radiation increased significantly only for six hours (intermittent) [3 hours on/1 hour off/3 hours on] compared to their negative control and sham groups (p < 0.05).

In this study, we did not find any differences in the gene expression levels of CASP8, CASP9, CASP3 and CASP12 of HT-29 cells exposed to 2100 MHz RF radiation for six hours (continuous) with respect to their negative control and sham groups (p > 0.05).

The gene expression level of CASP12 did not change under exposure to 2100 MHz RF radiation for all three exposure durations compared to their negative control and sham groups (p > 0.05) (Figs 7–10).
303 MHz RF radiation exposure

Cell viability

Significant decreases were found in the percentage rates of cell viabilities of HT-29 cells exposed to 2600 MHz GSM-like signals for all three exposure durations compared to their negative controls and sham groups (p < 0.05).

Genetic analysis

The gene expression levels of CASP8, CASP9, CASP3 and CASP12 did not change under exposure to 2600 MHz RF radiation for all exposure durations compared to their negative controls and sham groups (p > 0.05) (Figs 11–14).

Discussion

In this study, we intended to investigate the probable impacts of RFR exposure at different frequencies [2nd generation (1800 MHz GSM-like RF signal), 3rd generation (2100 MHz UMTS-like RF field) and New Mobile Phone 4th generation (2600 MHz]
LTE Signal Repeater) for different exposure durations (3 hours continuous, 6 hours intermittent, 6 hours continuous) on caspase-dependent cell death in human colorectal adenocarcinoma cell lines. Radiofrequency radiation has been suggested as a stress inducer that can activate and/or maintain various biochemical and physiological responses (French et al, 2001). And thus, we applied different RF signals as the probable stress factor to human colon cancer cells and analyzed gene expression levels of caspases: caspases involved in the extrinsic pathway (CASP8, CASP3), in the mitochondrial pathway (CASP9) and the endoplasmic reticulum stress-induced (CASP12) of cell death.

The homeostasis of colon epithelium, the balance between proliferation, senescence and death, can change under the influence of different internal and external factors resulting in the disruption of a signaling or the changes in the process of apoptosis. And mostly, these changes lead to the initiation and progression of an adenoma to carcinoma [colorectal cancer (CRC)] (Sancho et al, 2004).

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**Fig. 9.** The gene expression levels of CASP3 in HT-29 cells exposed to 2100 MHz RF radiation for 3 hours continuous, 6 hours intermittent (HT-29 cells were exposed to RF radiation for 3 hours continuous/1 hour HT-29 cells were held at incubator/HT-29 cells were exposed to RF radiation for 3 hours continuous) and 6 hours continuous. ••: RFR exposure Group vs Control and Sham Groups respectively (p<0.05)

**Fig. 10.** The gene expression levels of CASP12 in HT-29 cells exposed to 2100 MHz RF radiation for 3 hours continuous, 6 hours intermittent (HT-29 cells were exposed to RF radiation for 3 hours continuous/1 hour HT-29 cells were held at incubator/HT-29 cells were exposed to RF radiation for 3 hours continuous) and 6 hours continuous.

**Fig. 11.** The gene expression levels of CASP8 in HT-29 cells exposed to 2600 MHz RF radiation for 3 hours continuous, 6 hours intermittent (HT-29 cells were exposed to RF radiation for 3 hours continuous/1 hour HT-29 cells were held at incubator/HT-29 cells were exposed to RF radiation for 3 hours continuous) and 6 hours continuous.

**Fig. 12.** The gene expression levels of CASP9 in HT-29 cells exposed to 2600 MHz RF radiation for 3 hours continuous, 6 hours intermittent (HT-29 cells were exposed to RF radiation for 3 hours continuous/1 hour HT-29 cells were held at incubator/HT-29 cells were exposed to RF radiation for 3 hours continuous) and 6 hours continuous.
On defining the three distinct phases of carcinogenesis namely, the initiation, promotion, and progression stages, Radiofrequency (RF) Electromagnetic (EM) fields have been still investigated in detail as one of the environmental agents that have tumour promoting effect. Since quantum energies of RF radiation (RFR) are too low to be able to break the weakest intermolecular bonds, tumour promoting properties of these fields are mostly highlighted instead of their tumourigenic effect.

Radiofrequency/Microwave (MW) radiation interact with living systems in 3 main ways: i. Penetration into the tissue, ii. Their propagation into the living system, iii. The primary interaction of the waves with tissue and the possible secondary effects arising from the primary interaction (Rachael, 2010). Furthermore, the resonance effect of RF/MW radiation may lead to genetic or epigenetic damage by the local overproduction of reactive intermediates or “free radicals” (Bioinitiative Report, 2008)

RF/MW penetration into the living systems/tissue that induces the over-production of free radicals or reactive intermediates [reactive oxygen/nitrogen species (ROS/RNS)]. Free radicals or ROS/RNS are highly reactive intermediates and one of the internal initiating factors of the signaling mechanisms (Forman et al, 2014). In literature, the effect of radiofrequency radiation on reactive...
oxygen species has been shown as “an agent of ROS production” that induces negative cellular response in living organisms (Yakymenko et al., 2015, Dasdagh et al., 2008, Kesari and Behari, 2009, 2012). Physiologically, reactive oxygen species are produced in response to various cellular functions, whereas antioxidant defense mechanism/system in mammals protect specifically to structural biomolecules (DNA, protein, lipid) against oxidative damage induced by radical intermediates (Güler et al., 2010, 2012, Tomrük et al., 2010, Ozgur et al., 2010, 2013, 2015, Kismali et al., 2012, Şahin et al., 2016, Xu et al. 2010, Karaca et al. 2012). ROS-related damage has contributed to the development of several diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and others (Ames et al., 1993, Halliwell and Gutteridge, 1992). For example, in cancer cells, antioxidant activity should increase proportionally to ROS production for maintaining the cellular redox balance (Gorrini et al., 2013).

Cancer mechanisms can be evaluated in terms of both epigenetics and genetics. In the epigenetic process, combined effects of environmental factors do not directly affect and change genes, but genetic information can be modified by mutations in gene expressions which can lead to the development of cancer. In the literature, there are many scientific studies that show that RFR exposures at different frequencies can lead to genetic alterations or damage (single and double strand breaks, chromosomal aberrations, micronuclei, mutations, etc.) in different types of tissues (Lai and Singh, 1995, 1996, Nikolova et al., 2005.; Paulraj and Behari, 2006, Yao et al., 2008, Valbonesi et al., 2008, Schwarz et al., 2008, Samino et al., 2009, Kumar et al., 2010, Garaj-Vrhovac et al., 2011, Güler et al., 2012, Kesari et al., 2014, Gandhi et al., 2015; Deshmukh et al., 2016, Akdag et al., 2016, Sahin et al., 2016, Xing et al., 2016, Sun et al., 2017, Pandey et al., 2017).

In the present study, we found that the viable rates of HT-29 cells for 1800 MHz and 2600 MHz RF radiation exposure and for different exposure duration [3-h continuous, 6-h intermittent and 6-h continuous] were different from the findings of gene expression levels of CASP8, CASP9, CASP3 and CASP12. Although the viable cell rates decreased through the influence of prolonged exposure time, there were no differences in all gene expression levels we analyzed for all exposure duration. Actually, a reduction in the viable cell rate is characterized by both increased rates and gene expression levels. Reduced viable cell rates with exposure to 2100 MHz RF radiation was applied for 3 hours continuously and 6 hours intermittently. Generally, Caspase-8 then activates downstream effector caspases such as caspase-3, resulting in apoptosis. And the intrinsic apoptotic pathway is characterized by the activation of the apoptotic function of mitochondria, [the release of cytochrome c, and activation of caspase-9]. Following the activation of caspase-9 in the apoptosis the cleavage of caspase-3 occurs. In our study, Caspase-3 gene expression levels were, however, found to increase significantly only during 6 hours of intermittent exposure (Fig. 15)

Another pathway that triggers apoptosis is the endoplasmic reticulum stress dependent apoptotic pathway. (Nagakawa et al., 2000, Yoneda et al., 2001). An increased amount of intracellular free Ca²⁺ leads to the activation of calpain and the conversion of inactive pro-caspase-12 to active Caspase-12. Active Caspase-12 can mediate directly the activation of Caspase-3 that has been activated by the inactive pro-caspase-9 passed through the cytoplasm without cytochrome c (Fan et al., 2005). According to our results, it can be said that 2100 MHz RF radiation can activate both caspase-8 - death receptor pathway initiator caspase and caspase-9 – internal mitochondrial pathway initiator caspase and that the initiator caspases can activate caspase-3 after 3 hours of exposure. Yet, the fact that there was no change in gene expression levels of caspase-12 according to its own negative control and sham groups indicated that caspase-9 and caspase-3 activation was not leading to apoptosis due to endoplasmic reticulum stress. Lu et al (2012) also confirm our results, in this study, the authors show that exposure to 900 MHz GSM like RF radiation for different exposure duration (1, 2, 3, 4 h) can activate and increase caspase-3 level in human blood mononuclear cells (Lu et al., 2012). Zhao et al (2007) determine that exposure to 1900 MHz GSM modulated RF radiation for 2 hours upregulated Caspase-2, Caspase-6 and Asc (Zhao et al., 2007). Palumba et al (2008) found that a slight but statistically significant increase in caspase 3 activity occurs in Jurkat cells exposed to 900 MHz GSM signals for 1 hour (Palumbo et al, 2008). Zuo et al (2014) revealed that microwave radiation could induce neural cell apoptosis via the classical mitochondria-dependent caspase-3 pathway. According to their study exposure to 2.856 GHz MW radiation for 5 min and 15 min, apoptosis indicators (chromatin condensation, apoptotic body formation, the mitochondria membrane potential decreased, DNA fragmentation increased, the ratio of Bax/Bcl-2, expression of cytochrome c, cleaved caspase-3 and PARP all increased) increased in neural cells (Zuo et al., 2014).

In summary, apoptosis plays an active role in many pathological and physiological actions. Therefore, interfering in the apoptotic process and re-arranging it can bring new methods of treatment into discussion. Our study may be beneficial for other experiments and also further investigations on in vitro mechanism.
of RF radiation at different frequencies. Further studies are necessary to determine the precise mechanism of caspase-dependent apoptosis induced by radiofrequency radiation.

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