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Effect of Pulse-Modulated GSM-900 MHz Electromagnetic Field on the Electrochemotherapy Efficacy of 4T-1 Cells

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ABSTRACT

Introduction: Electrochemotherapy (ECT) is a cancer treatment modality to permeabilize cell membrane facilitating the non-permeant molecules to gain access to the cytosol of cells. Nevertheless, environmental electromagnetic fields (EMFs) may disturb the efficiency of ECT. The present study aimed to investigate the effect of EMFs 900 MHz pulse-modulated by 217 Hz extremely low-frequency fields on the efficiency of ECT

Materials and Methods: The 4T-1 cells were exposed to 900 MHz radiofrequency (RF) modulated by 217 Hz fields at the power densities of 17, 162, and 349 μW/cm^2 (related to antenna input powers of 3, 4, and 5 W at a distance of 15 cm) by a GSM900 MHz simulator. After exposure, the cells were divided into several groups, receiving no treatment, chemotherapy, electric pulse, and ECT. The cell viability was evaluated by MTT assay after 24 h.

Results: The results demonstrated that 900 MHz RF pulse-modulated by 217 Hz EMF at 349 μW/cm^2 increased the viability of the cells treated with EPs with the amplitude of 70 V/cm and frequency of 5 kHz (16%), ECT with 70 V/cm at 5 kHz (20%), and ECT with 60 V/cm at 5 kHz (16%), compared to their counterpart treatment group with no exposure. However, the fields had no significant effect on the efficacy of chemotherapy.

Conclusion: As the findings of the current study indicated, environmental pulsed-modulated RF fields exerted an adverse influence on some antitumor therapies. Therefore, such effects should be taken into consideration in determining the optimal protocols of treatment.

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Introduction

Nowadays, cancer as an epidemic disease is considered as an important cause of human mortality. The novel techniques are subjected to many investigations targeted toward the identification and improvement of cancer treatment. Electrochemotherapy (ECT) is one of the therapeutic methods that utilizes electric pulses (EPs) to facilitate the permeabilization of the cell membrane to poorly-permeant anticancer drugs like bleomycin [1]. The cells die as a result of DNA breaks caused by bleomycin [2]. Any disturbance in the treatment mechanism can make this process difficult. The disturbance may occur due to many confounders, even environmental agents.

Cell phones are among the environmental factors, which have attracted the researchers' attention to investigate their biological effects. Cell phones emit non-ionizing electromagnetic energy within a radiofrequency (RF) of about 900 MHz [3, 4]. Many studies have shown that mobile phone electromagnetic fields (EMFs) may affect some of the biological functions of human [5-7]. Moreover, EMFs induce some changes in the cytotoxicity of antineoplastic drugs. In other words, the cells pre-exposed to EMFs may get resistant to the harmful effects induced by subsequent cytotoxic agent, thereby making the treatment of malignant tumors difficult.

Resistance to the damage induced by the genotoxic agent through pre-exposure to EFMs is termed as "adaptive response" [8-10]. In this regard, EMFs could be considered as agents, which influence DNA molecule as a biological target and decrease treatment efficacy through rendering an adaptive response. In other words, EFMs can modulate DNA repair process in the cells [10] and lead to the reduction of the toxicity of the following genotoxic agents. Accordingly, several studies have evaluated the decreased percentage of cancer cell death in the cells treated by genotoxic agents and pre-exposed to RF fields [10].

In addition, the results obtained in our earlier study revealed that the pre-exposure of 4T-1 to 900 MHz RF radiation resembling the radiation emitted by mobile phones had a protective effect on the cell death induced by the following ECT [11]. However, the signals in GSM mobile phones are 900 MHz waves

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modulated by rectangular pulses with the frequency of 217 Hz related to the domain modulation component in GSM mobile phone systems.

Regarding this, it is necessary to investigate whether the modulation of amplitude change is the result of the cancer cell exposure to 900 MHz alone or not. With this background in mind, the aim of the current study was to examine the effect of the pre-exposure to 900 MHz RF fields modulated by 217 Hz extremely-low frequency (ELF) resembling the radiation emitted by GSM-900 MHZ mobile phones - on the protective response of 4T-1 cells (mouse breast cancer cell) treated with chemotherapy, EPs, and ECT.

There are some studies investigating the protective effects of RF waves against such treatments as chemotherapy or ionizing radiation [10, 12]. However, to the best of our knowledge, the role of EMFs emitted by GSM mobile phones in changing the efficacy of treatment with EPs has not been studied yet. The examination of the effect of these fields on ECT could be of significant importance due to the advantages of this therapeutic approach, in comparison to the other treatments, such as radiotherapy and chemotherapy. Therefore, the findings of this study could be helpful in taking the first step in the identification of the optimal ECT protocol with higher efficacy in cancer treatment.

Materials and Methods

This experimental study was conducted on the cancer cells of 4T-1.

Cell culture

In this study, we used 4T-1 mouse mammary tumor cells due to the higher applicability of EPs in the treatment of superficial tumors, such as breast cancer. The cells were cultured in a complete medium, including RPMI, 10% fetal bovine serum, and 1% penicillin-streptomycin at 37°C using a 5% CO_2 humidified incubator. Sub-culturing was carried out every two days.

Radiation exposure

The exposure system was a 900-MHz GSM homemade simulator emitting 900 MHz RF pulse, modulated by 217 Hz signals similar to those, generated by mobile phones. Power density was measured by a portable electromagnetic field monitoring system named wave-control (SMP2, Spain) at a distance of 15 cm to obtain the intended amount. The measurement mode was set on ICNIRP (International commission on Non Ionizing Radiation Protection) mode (Measurement time: 6 min, log interval: 0.5 sec). In line with a study performed by *Jadidi et al.*, the transmitted powers to the simulator antenna were 3, 4, and 5 W [13, 14]. Furthermore, the power densities acquired in the distance of 15 cm around the antenna (in the middle of the distance and between the near field and far field of antenna) were 17, 162, and 349 $\mu W/cm^2$ (equivalent special absorption rates of 0.01, 0.08, and 0.5 W/kg, respectively).

Subsequently, 1×10^5 cell/ml were transferred to a small Petri dish and set in the predetermined location (i.e., the distance of 15 cm around the antenna) and exposed to RF waves pulse-modulated by 217 Hz fields in the mentioned power densities for 10 min. The temperature was measured before and after the experiment. The measured temperature did not vary in the cell culture medium [15].

Treatments

After exposure of the cells to modulated RF waves (at 17, 162, and 349 $\mu W/cm^2$), the experimental groups were treated as follows:

A) Four groups were exposed to pulse-modulated RF waves (at 0, 17, 162, and 349 $\mu W/cm^2$).

B) Four groups were exposed to pulse-modulated RF waves (at 0, 17, 162, and 349 $\mu W/cm^2$), and then treated with chemotherapy.

C) Twelve groups were exposed to pulse-modulated RF waves (at 0, 17, 162, and 349 $\mu W/cm^2$), and then treated with EPs (4,000 pulses, 100 ms square wave EPs with three different protocols, namely amplitude of 70 V/cm and frequency of 5 kHz, amplitude of 60 V/cm and frequency of 5 kHz, and amplitude of 70 V/cm and frequency of 4 kHz).

D) Twelve groups were exposed to pulse-modulated RF waves (at 0, 17, 162, and 349 $\mu W/cm^2$), and then treated with ECT (4,000 pulses, 100 ms square wave EPs with three different protocol, including amplitude of 70 V/cm and frequency of 5 kHz, amplitude of 60 V/cm and frequency of 5 kHz, and amplitude of 70 V/cm and frequency of 4 kHz).

For EP and ECT, EPs with the low frequency and high voltage were generated by a pulse generator (Pulse Porator, Tehran, Iran). The prepared cell suspension was subjected to uniform electric field through stainless steel electrodes. The concentration of bleomycin drug for chemotherapy and ECT was 1 µM. For ECT, bleomycin was added into the cells, and then the pulses were applied. After treatments, the cells were incubated for 24 h. All experiments were repeated more than three times.

MTT assay

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay was carried out 24 h after treatments to measure the cell viability. After discarding the culture medium, 10 µl of MTT solution (5 mg/ml) in phosphate buffered saline was added to the cells in each well, and the cells were incubated at 37°C. After 4 h, the supernatant was removed, and 100 µl dimethyl sulfoxide was added to the cells. Finally, the density was read at 540 nm using the Multiscan MS ELISA reader (Labsystems Multiscan MS, UK). The cell viability results were depicted as percentages in comparison with the control group.

Statistical analysis

The cell viability assays were presented as mean and standard deviation. Comparisons between the groups were conducted using the repeated measures ANOVA. Data analysis was performed in SPSS software (version 16). P-value less than 0.05 was considered statistically significant.

Results

The experiments were performed to clarify the alternations in the cell viability under the influence of a radiation similar to that emitted by mobile phones and some cancer treatments.

Electromagnetic field exposure alone

The results of the cell viability after exposure to radiation without any treatment are illustrated in Figure 1. According to the results, there was no significant change in 4T-1 cell viability, indicating that the fields could not kill the cells during the 10-minute exposure. However, the cells exposed to EMF demonstrated a slight decrease in cell viability, compared to the sham group, especially at 17 μW/cm^2. The enhancement of radiation exposure, more cell death.

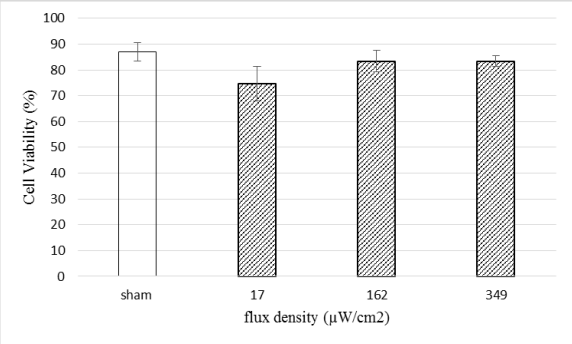


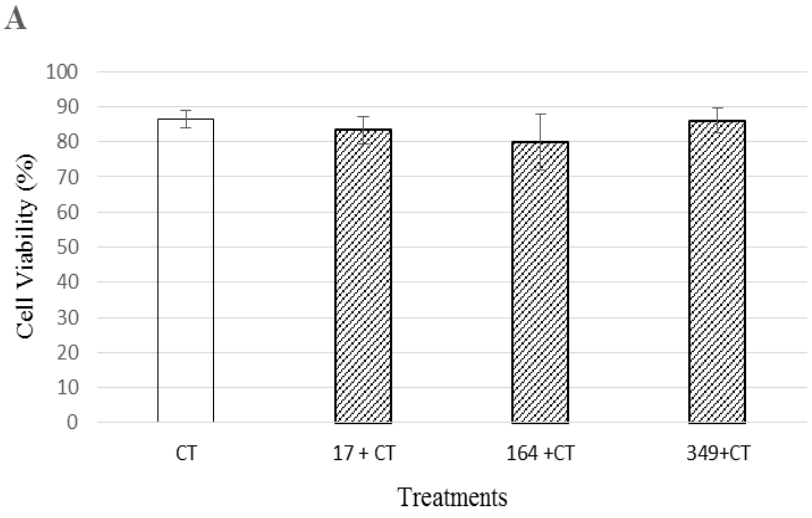
Figure 1. Effect of radiofrequency radiation modulated by 217 HZ at power densities of 17, 162, 349 μW/cm^2 on cell viability; white column: percentage of the viable cells under the experiment conditions, while the non-radiation exposure was normalized to the control group; pattern filled columns: percentage of the viable cells in the radiation-exposed cells expressed as the percentage of control. Data are displayed as a mean of at least three separate experiments ± standard deviation. Each experiment was performed in triplicate. Data are displayed as a mean of at least three separate experiments ± standard deviation. Each experiment was performed in triplicate.

Chemotherapy after Exposure to Electromagnetic Fields

In the chemotherapy groups, the pre-exposure of the cells to radiation did not change the cell viability. Therefore, no resistance to bleomycin resulting from EMFs was observed. Nonetheless, there were insignificant reductions of 3% and 6% at 17 and 162 μW/cm, respectively in the cells pre-exposed to radiation and then treated by chemotherapy as compared to those subjected to chemotherapy alone, which indicated a negligible enhancement of the chemical susceptibility of the cells to chemotherapy

Electric pulse and electrochemotherapy in 4T-1 cells following electromagnetic field exposure

In the EP groups, a significant difference was observed between the group pre-exposed to EMF at the power density of 349 μW/cm^2 and then treated by EPs with a frequency of 5 kHz and amplitude of 70 V/cm (nearly 16%) and the group treated by EP (70 V/cm, 5 kHz) alone in terms of the percentage of the viable cells. The difference demonstrated a protective response to EP at 349 μW/cm^2. No significant protective response was observed in the other groups. However, the percentage of cell viability under the treatment after exposure to 162 μW/cm^2 was lower than that of the other groups. In other words, the power density of 162 μW/cm^2 caused higher 4T-1 cell susceptibility to EPs



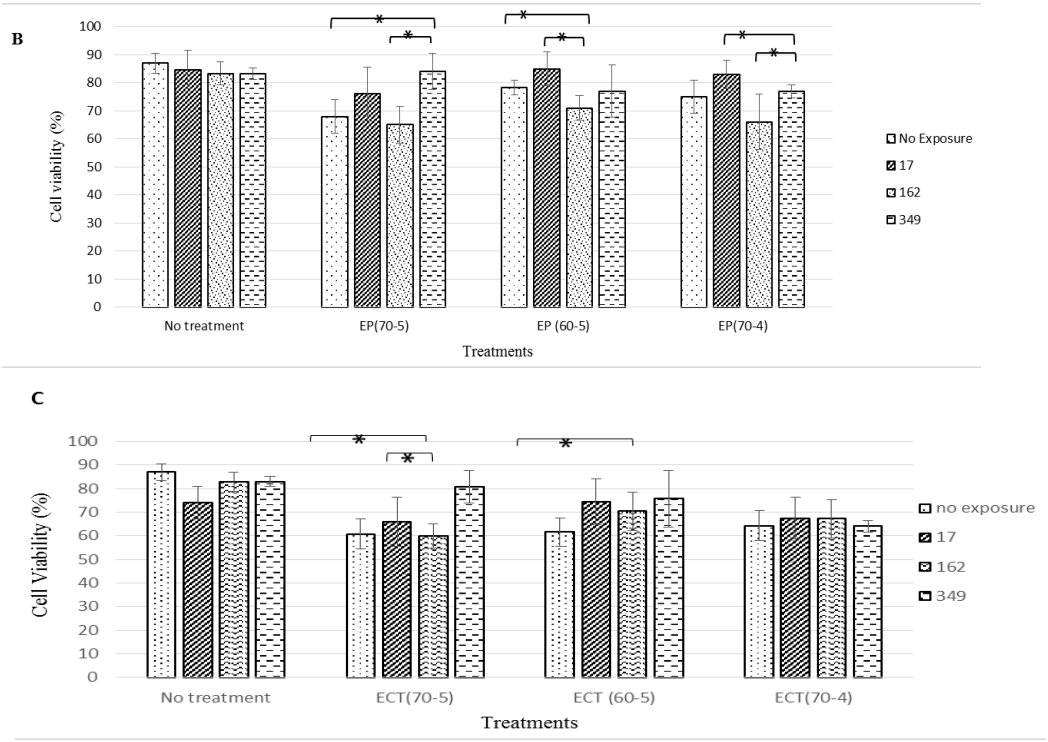


Figure 2. (A): percentage of the viable cells for treatment by chemotherapy after exposure to radiation with the power densities of 17, 162, and 349 μW/cm^2; (B): percentage of the viable cells for treatment by magnetic fields exposure alone, electric pulses alone, and treatments (the three protocols of electric pulses included 70 V/cm at 5 kHz, 60 V/cm at 5 kHz, and 70 V/cm at 4 kHz) after exposure to radiation with the power densities of 17, 162, and 349 μW/cm^2; (C): percentage of the viable cells for treatment by magnetic fields exposure alone, electrochemotherapy alone, and treatments (the three protocols of electrochemotherapy included 70 V/cm at 5 kHz, 60 V/cm at 5 kHz, and 70 V/cm at 4 kHz) after exposure to radiation with the power densities of 17, 162, and 349 μW/cm^2. Data are displayed as a mean of at least three separate experiments ± standard deviation. Each experiment was performed in triplicate. Statistical significance was determined by the Student's t-test analysis at *P<0.05.

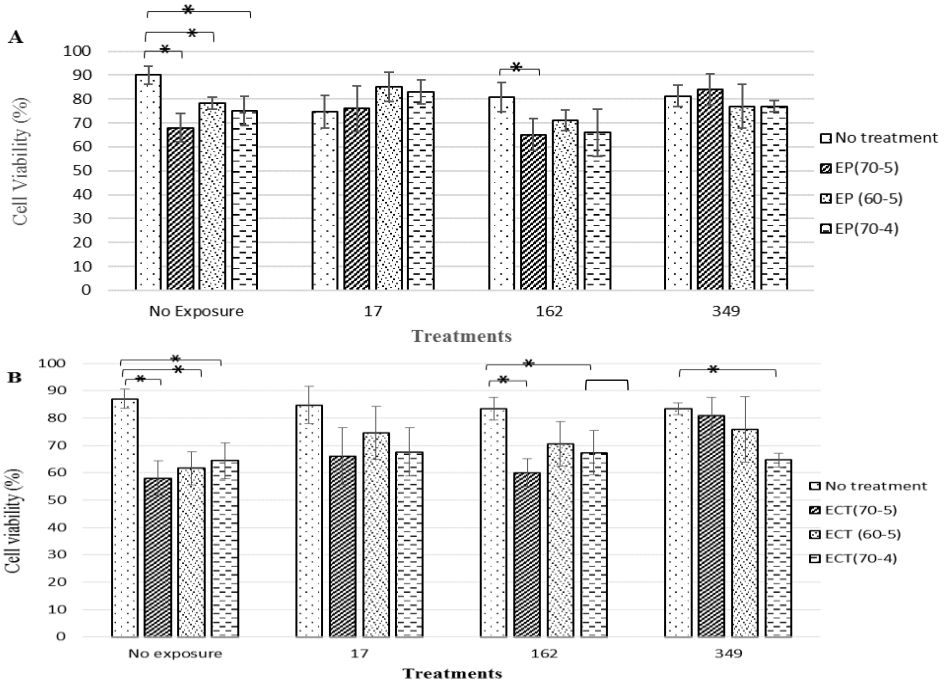


Figure 3. Effect of radiofrequency radiation modulated by 217 Hz on the efficacy of electric pulses or electrochemotherapy treatments; (A): percentage of the viable cells for treatment by electric pulses alone and treatments (the three protocol of electric pulses included 70V/cm at 5 kHz, 60 V/cm at 5 kHz, and 70 V/cm at 4 kHz) after exposure to radiation with the power densities of 17, 162, and 349 μW/cm2; (B): percentage of the viable cells for treatment by electrochemotherapy alone and treatments (the three protocol of electrochemotherapy included 70V/cm at 5 kHz, 60 V/cm at 5 kHz, and 70 V/cm at 4 kHz) after exposure to radiation with the power densities of 17,162, and 349 μW/cm2. Data are displayed as a mean of at least three separate experiments ± standard deviation. Each experiment was performed in triplicate. Statistical significance was determined by the Student's t-test analysis at P<0.05.

In the ECT groups, the power density of 349 μW/cm^2 could increase the percentage of the viable cells after the implementation of ECT with a frequency of 5 kHz and amplitude of 70 V/cm, compared with ECT alone (70 V/cm at 5 kHz; 19%) . This enhancement was also observed after performing ECT with an amplitude of 60 V/cm and frequency of 5 kHz, compared with ECT alone with 60 V/cm at 5 kHz (16%). The groups treated with ECT at the power density of 349 with 70 V/cm at 5 kHz and 60 V/cm at 5 kHz had the highest cell viability percentage among the other groups.

The power density of 17 μW/cm^2 led to an insignificant increase in the viability of the cells treated with EP or ECT, compared with the treated cells without exposure. Nevertheless, although the power density of 162 μW/cm^2 decreased the cell viability in chemotherapy, and also in the protocols of EP (70-5), compared to EP alone (70 -5), a similar decrease was not observed for ECT (70-5). Analogous results were obtained for the other protocols (i.e., EP [60-5], EP [70-4]). According to the results, pulse-modulated RF exposure alone had an effect on the damage induced by EP or ECT. **]**

Discussion

There are well-documented scientific evidence indicating the protection of the cells pre-exposed to EMFs against the toxic effects of some genotoxic agents [8-10, 12, 16]. These pieces of evidence highlight the role of environmental EMFs in the efficacy of cancer therapeutic modalities. Consequently, the researchers should direct considerable attention to the effects of EMFs in determining the optimal treatment protocols. In this regard, the results of our previous study demonstrated that 900 MHz RF radiation was capable of inducing a protective effect against chemotherapy, EP, and ECT [11].

The current study investigated the impact of pre-exposure to EMF 900 MHz frequency modulated by 217 Hz in the efficacy of the mentioned treatments to observe the role of modulation of RF waves. Prior to the interpretation of the results, it should be noted that any temperature increase was not observed within the Petri dish containing the cells. Therefore, the recorded effects should be considered as non-thermal caused by the radiation.

The results regarding the effects of EMFs on chemotherapy showed that the pulse-modulated RF pre-exposure could not protect the 4T-1 cells from the toxic effects exerted by bleomycin. This finding is in contradiction with a study, reporting a decrease in the toxic effects of chemotherapy drugs resulting from RF exposure [10]. This discrepancy could be attributed to two factors, namely the difference in the exposure duration of the two studies and the difference in cell type (4T-1 in our study and HI-60 in the study of Jin et al.). Therefore, these two factors may lead to different cell responses to chemotherapy as a result of EMFs pre-exposure.

The different cells with various properties may have unlike responses to stressors. For instance, in the current study (figures 2, 3), 4T-1 cell death caused by EP (nearly 75%) and ECT (almost 60%) alone in low frequency and high voltage was less than that in MCF-7 cells in the study of Shankayi et al. (EP: nearly 55%, ECT: almost 30%) [17]. The difference could be due to the diversity of the cell size, shape, structure or subtype.

One considerable result was the observation of the slight sensitivity of the cells to EP and chemotherapy at 162 μW/cm^2 in comparison to two other power densities. In spite of the reduction of the cell viability in these two treatments, there was no decrease in the cell viability caused by ECT at the power density of 162 μW/cm^2. In other words, although ECT is the combination of EP and chemotherapy, EMF exerted a different effect on cell endpoint in ECT, compared to that in EP and chemotherapy. Accordingly, the mechanism by which EMFs influence cell chemical sensitivity to chemotherapy or cell electrical sensitivity to EPs seems to be different from that of EMFs affecting cell sensitivity to the combination of drug and EPs.

The results pertaining to the variations in the cell viability did not show an upward or downward trend with the enhancement of power density. Nonetheless, an analogous trend was observed in changes of the cell viability for the cells exposed to radiation and then treated by EP (70 V/cm amplitude and 5 kHz frequency or 60 V/cm amplitude and 5 kHz frequency) at the power densities of 17 and 162 μW/cm^2. The trend was that the cell viability increased at 17 μW/cm^2 and decreased at 162 μW/cm^2, compared to that treated with EP alone. Window effect [18] can describe this non-linear trend.

However, with regard to the resistance to treatment, consistent with the literature, the same protective response was found at 349 μW/cm^2 so that the cell viability increased in two protocol of ECT, namely 70 V/cm amplitude and 5 kHz frequency (nearly 20%), as well as 60 V/cm amplitude and 5 kHz frequency (almost 14%). There was no change in the cell viability when the cells were pre-exposed to EMFs at 349 μW/cm^2 and then treated with ECT with the frequency of 4 kHz and amplitude of 70 V/cm.

The results indicated that the frequency of EPs executed a more important role in EMF effects on EP and ECT efficacy than the voltage of EPs. Moreover, in our previous study, the unmodulated RF radiation at the power density of 162 μW/cm^2 was found to be more effective in providing a protective response to death induced by ECT [11]. However, in the current study, the response was seen at 349 μW/cm^2. Therefore, it seems that the modulation of amplitude may change the effectiveness window of RF-EMFs.

The results related to the effects of EMF 900 MHz frequency modulated by 217 Hz at the power density of 349 μW/cm^2 showed an increase in cell viability in both EP (70-5) and ECT (70-5). Because EP results in an irreversible damage to the membrane of the cells, EMFs may increase the viability of the cells treated by EP (70-

5) alone through affecting some electrical characteristics of the cell membrane, including polarization, permeability, and conductivity.

However, ECT leads to damages in DNA and cell membrane induced by bleomycin action and EPs, respectively. Therefore, EMFs also affect DNA damage induced by bleomycin, which enters the cells permeabilized by EPs. The mechanisms leading to the entrance of bleomycin into the cells through low-voltage EPs may be influenced by EMFs, thereby decreasing ECT efficacy.

Even though there is no explanation regarding the observation of significant results related to the protective effects of EMFs, there are some laboratory studies, demonstrating that EMFs alter some biological processes. Resistance to the treatment could be the result of DNA repair, which may be induced by EMFs. The development of such resistance probably occurs in several ways. DNA repair is strongly influenced by changes in cytosolic ion concentrations (particularly calcium), and the changes can be induced by EMFs [19, 20].

Furthermore, magnetic fields may lead to the overproduction of heat shock protein involved in improving the efficiency of the DNA repair process [21]. In addition, increase in the expression of genes, encoding DNA repair proteins, is proposed for the induction of protective effects [22]. In this study, the cell viability was investigated after two mitoses; the cells could repair DNA damage induced by the treatments during this time. Consequently, in addition to the contribution of the changes induced by EMFs to DNA repair, the mechanisms after treatment also assist to this process; therefore, the protective response was obtained more clearly.

However, in exposure to ELF pulse-modulated RF waves, the cells can sense both RF frequency and ELF frequency. In ECT and EP treatments using a low voltage, the cells are killed through the apoptosis process [23, 24]. Therefore, it can be supposed that the resistance mechanisms developed by ELF fields (related to the modulation of amplitude in the EMFs) may prevent cell apoptosis and cause cell viability to increase.

There are mechanisms that could give rise to a cellular defense against the effect of stressors. These mechanisms include enhanced Ca^{2+} influx from extracellular space by ELF magnetic fields [25], increased antioxidant enzymes activity after exposure to the ELF magnetic fields [26], and elevated levels of BAG3 anti-apoptotic protein induced by ELF-MF [27]. Nevertheless, the involvement of different defense mechanisms in the minimization of the induced genotoxic damage makes it difficult to identify the exact mechanism of the protective response developed by EMFs in the therapeutic approaches using a genotoxic agent.

Conclusion

As the findings of the present study indicated, pulse-modulated EMFs similar to those emitted by mobile phones can result in the development of resistance to cell death induced by ECT at a specific power density. This protective response was observed at EMFs of 349 $\mu W/cm^2$ in the two protocols (i.e., 70 V/cm at 5 kHz and 60 V/cm at 5 kHz) used for ECT. From a clinical point of view, the results highlighted the importance of examining the weakened effects of environmental EMFs in antitumor therapies. Therefore, this issue need further exploration of the mechanisms and processes of treatments influenced by EMF through the implementation of in vivo studies.

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