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Effects of 1Hz 100mT Electromagnetic Field on Apoptosis Induction and Bax/Bcl-2 Expression Ratio in Breast Cancer Cells

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Introduction: Breast cancer is the most common metastatic malignancy and the second leading cause of cancer death in women. Recently, extremely low-frequency electromagnetic fields (ELF-EMFs) seem to modulate the rate of proliferation and enhance apoptosis and are considered as an emerging approach to cancer therapy. Despite recent success in the electromagnetic fields, the results are still neither definitive nor even contradictory.

Methods: In this study, induction of apoptosis was considered as one of the possible mechanisms of ELF-EMFs on cancer inhibition. Breast cancer cell lines were exposed to a 1 Hz, 100 mT ELF-EMF (2 h/day) for five days. The apoptosis rate of both the exposure and sham exposure groups was determined by flow cytometry. The expression levels of Bax and Bcl-2 were evaluated by real-time PCR.

Results: The mRNA expression levels of Bax were increased; while the expression of Bcl-2 showed a decrease in MDA-MB231 cells exposed to 1Hz EMF compared with sham exposure. Moreover, the ratio of Bax/Bcl-2 was significantly increased in comparison to the sham exposure. The increased Bax/Bcl-2 ratio induces cell apoptosis.

Conclusions: It is suggested that ELF-EMF is a new adjuvant therapeutical method that may contribute to anti-cancer and cancer therapy research.

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INTRODUCTION

Cancer is a debilitating disease that affects a considerable portion of the world's population across all generations and is a global public health problem [1]. Among various types of cancer, breast cancer is the most common metastatic malignancy and the second leading cause of cancer death in women all around the world [2, 3]. Despite the impressive progress made in conservation methods and recent advances in screening and chemotherapy techniques, no definitive therapy has been found for the advanced stages of this disease [4]. The cell apoptosis process is of considerable interest in oncology and cancer therapy; indicating a common molecular pathway in carcinogenesis drug resistance [5]. Apoptosis as a form of programmed cell death (PCD) is a conserved gene-controlled method used to eliminate unwanted or unnecessary cells in living organisms which is also essential for

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tissue development and homeostasis [6]. Apoptosis is observed in many defensive mechanisms of the immune system or the course of diseases. Any disturbance in this process may cause illness which can be due to a reduction in cell death, an increase in cancer cell growth, or weakness of autoimmune disorders [7]. Electromagnetic fields with a frequency of less than 300 Hz are known as extremely low-frequency electromagnetic fields (ELF-EMFs). These fields are non-invasive and non-ionizing which have even non-thermal effects on cells and tissues [8]. These characteristics have led to the study of the effect of ELF-EMF on the evolutionary path of various diseases including cancer. Over recent years, the beneficial effects of ELF-EMF on different cancer cells have been shown in several studies. A large number of studies have shown that the low frequency and energy of EMF can affect different biological processes such as apoptosis, cell proliferation, and gene expression [9-14]. Despite recent success in this field, the results are still neither definitive nor even contradictory [15, 16]. Therefore, according to the obtained results from previous studies, we investigated the induction of apoptosis and changes in the expression level of important involved genes after exposure to an ELF-EMF within a specified time interval in the triple-negative breast cancer cell line.

METHODS

Cell Culture

The triple-negative human breast cancer cell line (MDA-MB-231) was obtained from the Cell Bank of the Iranian Institute of Genetic Resources (Iran). Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA); containing 10% heat-inactivated fetal bovine serum (Gibco, USA), 100 µg/mL streptomycin, and 100 U/mL penicillin (Invitrogen, Burlington, ON) was used to culture MDA-MB-231 cells in 5% CO2 at 37°C. The cells were then seeded with a density of 5×104 cells/well in six-well plates.

EMF Exposure

An ELF-EMF generator has been used in this study as reported in our previous research [17]. The EMF generator machine has been constructed of two square coils with a U-shape solenoid-like configuration and an ironic core inside to provide homogenously and maximized B-field exposure. The side length of each stated coil is equal to 2 cm which is covered by 1250 single-strand copper wire. Coils were positioned in a way that their shortest end-to-end distance was 15 cm. The coils were connected to a wave generator (GW Instek SFG-1000 Series, South Korea) with a tunable magnetic field at the intensity range of 1–100 mT and the frequency range of 1–60 Hz. For ELF-EMF exposure, 1 Hz ELF-EMF with an intensity of 100 mT was applied to the cells 2 h/day for 5 consecutive days. The same position was considered for simultaneous culturing of the sham exposure group with the experimental samples under identical environmental conditions in the same incubator but without magnetic fields.

Quantitative Real-Time PCR

The cells were harvested after treating with 1 Hz-EMFs and the total RNA was extracted by RNX reagent (Sina Clon, Iran). The RNA samples were frozen at −80°C until further analysis. The synthesis of cDNA was carried out; using a reverse transcriptase kit (Vivantis, Malaysia). The mRNA expression was analyzed in the following genes; using Quantitative real-time PCR (q-PCR) on an ABI 7500 amplification system (Applied Biosystem, USA). SYBR Green qPCR Master Mix Kit (Vivantis, Malaysia) and fold change= $2^{-\Delta\Delta Ct}$ method [18] were applied and normalized to β-actin expression. The sequences of the applied primers are listed in Table 1.

Flow Cytometry

MDA-MB231 cells were plated at a density of 106 cells/well in 24-well cell culture plates. After 24 hours of incubation, they were exposed to ELF-EMF

Table 1: Primers Used in the Real-Time Quantitative Polymerase Chain Reaction

Seq Name	5'-3' Forward	5'-3' Reverse
Bax	CAAACTGGTGCTCAAGGCC	GGGACATCAGTCGCTTCAGTG
Bcl-2	AGAGCGTCAACCGGGAGAT	ATCCCAGCCTCCGTTATCCT
β-actin	CTCCATCCTGGCCTCGCTGT	GCTGTCACCTTCACCGTTCC

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(100mT, 1 Hz for 5 days, 2 hours each day). At the end of the exposure period, the MDA-MB231 cells were collected and then stained; using the Annexin V-FITC (Malaysia, IQ product) apoptosis detection kit by the manufacturer's instructions. Finally, cell apoptosis was analyzed by a flow cytometer (BD FACS Calibur, USA).

Statistical Analysis

Graph pad Prism software version 6.01 (GraphPad Software, CA, USA) was employed for all statistical analyses. The t-test was performed for comparing the groups, and $P<0.05$ were assumed significant. All experiments were performed in at least duplicate.

RESULTS

Effects of 1Hz ELF-EMF on the Cell Apoptosis in MDA-MB231 Cells

According to the results of flow cytometry graphs, the quantitative analysis of apoptosis revealed that 1Hz ELF-EMF exposure with 100mT significantly promoted cell apoptosis in MDA-MB231 cells (8% $P<0.05$); compared to the sham exposure (Figure 1).

Effects of 1Hz ELF-EMF on Critical Gene Expression of Apoptosis in MDA-MB231 Cells

The quality of extracted RNAs was investigated on a 1% agarose gel electrophoresis (Figure 2). Then, we performed the real-time PCR assay to detect the gene expression of Bax and Bcl-2 at the messenger RNA (mRNA) level in MDA-MB231 cells subjected to 1Hz ELF-EMF stimulation (Figure 3). According to the results of the real-time PCR, EMF exposure with 100 mT induced a significant decrease in Bcl-2 (0.64-fold); while increasing the Bax (2.4-fold) and Bax/Bcl-2 ratio (3.7-fold) ($P<0.05$); compared with the sham exposure.

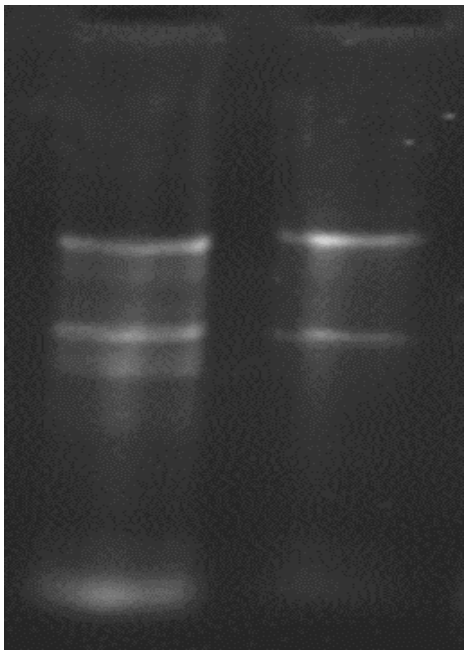


Figure 1: Electrophoresis of Extracted RNAs for Sham and Exposure Samples

DISCUSSION

Breast cancer is the uncontrolled growth and malignant proliferation of epithelial cells in breast tissue [19]. Recently, EMF seems to modulate the rate of proliferation and enhance apoptosis and is considered an emerging approach in cancer therapy. ELF-EMF has been shown to affect important cell processes, including apoptosis in cancer cells of the breast [20-22]. However, further research is needed to find possible mechanisms underlying this process. In our study, the MDA-MB-231 cell line was investigated in two groups of exposure

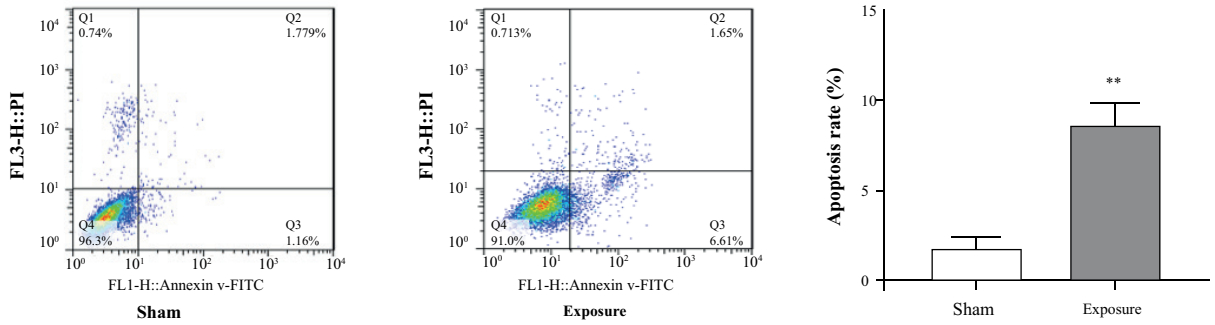


Figure 2: The Apoptosis Measurements of Extremely Low-Frequency Electromagnetic Fields (ELF-EMFs) Exposure and Sham Exposure by Flow Cytometry Data

Q1, population of necrotic cells (AnnexinV - /PI +); Q2: population of late apoptotic cells (AnnexinV + /PI +), Q3, population of early apoptotic cells (AnnexinV + /PI -); Q4: Population of live cells (Annexin V - /PI -)

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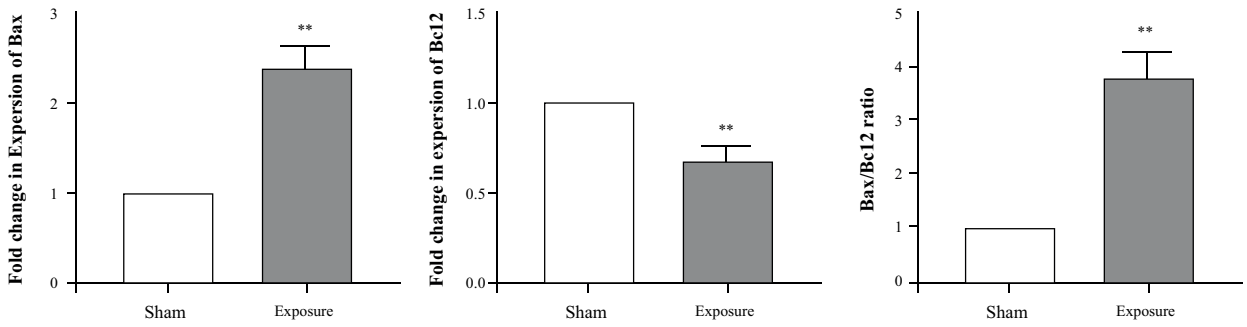


Figure 3: Extremely Low-Frequency Electromagnetic Fields (ELF-EMF) Exposure Promotes Apoptosis in MDA-MB231 Cells Quantitative real-time polymerase chain reaction analysis following 10h EMF exposure. Cells were examined for expression of Bax, Bcl-2, and Bax/Bcl-2 ratio without (Sham) or with (EMF) exposure to an EMF-ELF with 1 Hz (100 mT, 5 days, 2 hours each day). Data were presented as mean ± standard deviation. **P<0.01 (unpaired t-test).

and sham exposure. The rate of apoptosis was determined in exposure (100 mT, 5 days, 2 hours each day) and sham exposure groups; using the flow cytometry technique. Changes in Bax and Bcl-2 expression levels were examined to further clarify the effect of ELF-EMF on important genes in the cell apoptosis process. Several research studies have shown that EMFs can affect important cellular processes, namely apoptosis in cancer cells [23, 24]. However, there is relatively little consensus on the biological effects of EMF. This is partly due to the diverse characteristics of EMF (frequency, intensity, and time of windows) in different experiments and somehow due to the complexity of cellular responses to EMFs [25, 26]. In this study, we exposed cells to ELF-EMF (100mT/1 Hz) 2 hours a day for 5 days. Our results showed that among the above-mentioned characteristics in this range of electromagnetic fields, the apoptosis rate increased by ~7% in the exposure group compared to the sham exposure group. In a study by Tatarov et al., 1HZ, 100-mT EMF was used on the EpH4-MEKBcl2 cell line for 360 minutes per day for 4 weeks to investigate tumor growth and necrosis. Using the imaging system, they reported a significant reduction in tumor growth in vivo [27]. Simko et al., observed an increase in the induction of apoptosis in a human squamous cell carcinoma cell line (SCL II) after exposure to EMF (1 mT/50 Hz) [28]. Similar results were obtained in a study on the MCF-7 cell line and normal MCF-10 cell line; using a frequency of 20 Hz and the 2, 3, and 5 mT EMF for 60 minutes within 3 days. Cytotoxic effects were observed on the MCF-7 cell line but no changes were observed on the normal MCF-10 cell lines [29]. Moreover, in research on osteosarcoma cells (MG-63), it was found that EMF (1mT, 50HZ) can induce apoptosis [30].

In 2018, Yadamani et al., found that EMF (4mT, 50 Hz) induces apoptosis in TUBO transplant breast cancer mice. This therapy led to a significant reduction in tumor mass, induction of apoptosis, and decreased number of blood vessels [31]. Other experiences have shown that cancer cells that are simultaneously exposed to EMF (0.4-1 μT, 60Hz) and dexamethasone would experience an increase in PCD; while exposure to a 2 μT EMF with a frequency of 50Hz did not have such significant effects on cancer cells' cycles [32]. Some recent reports investigated the effect of EMF on MC4L2 cancer cells. Cells were exposed to a 100mT EMF with a frequency of 1Hz 2 hours per day for 5 days. About 35% of cancer cells were killed as they were exposed to this electromagnetic field [33]. Additionally, other studies were conducted on the effect of EMFs with a frequency of 50 Hz (20 mT, for 3 hours per day for up to four days) on the MDA-MB-231 breast cancer cell line. In this study, the fluorescent staining revealed that daily exposure of ELF-EMF induced apoptotic cell death in MDA-MB-231 with a decreased viability of MDA-MB-231; compared to the non-exposed group [34]. In a study by Ruiz Gomez et al., cell cycle distribution and apoptosis were investigated; using a 1.5 mT EMF with a frequency of 25 Hz for 2 hours within 45 minutes. According to the obtained results, no significant change was observed in the stages of the cell cycle and apoptosis induction [35]. Apoptotic cell death occurs in response to environmental stimuli. Regulation of apoptosis is of great importance for cancer treatment and normal growth as well as embryogenesis. Human histological studies and animal experimental models have shown that the Bcl-2 family (B-cell lymphoma2) regulates apoptotic

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cell death. In comparison to Bax (Bcl2-associated x protein) with pro-apoptotic properties, Bcl-2 is an anti-apoptotic molecule. Bcl-2 contributes to regulating apoptosis and codes different proteins that play a key role in cell apoptosis regulation. The BAX gene is known as a pro-apoptotic gene with similar functions as Bcl-2. Interaction between the members of the Bcl-2 family determines cell survival or death both in cytosol and mitochondria. Bax/Bcl-2 ratio has been found to determine the cell death or survival; following an apoptotic stimulus [36, 37]. Therefore, Bax/Bcl-2 expression ratio can strongly indicate cell apoptosis [38].

In our study, the expression of both Bax and Bcl-2 genes was investigated in both groups of exposure and sham exposure. Both genes showed significant changes in mRNA expression level after exposure to ELF-EMF; compared to the sham group. Accordingly, the expression level of Bcl-2 showed a significant reduction compared to the sham group. Additionally, the expression level of the Bax gene and Bax/Bcl-2 ratio increased significantly in comparison to the sham group. Our results agree with those obtained by Pan Wang in 2018; using a 15Hz EMF for 2 hours with an intensity of 30 Gauss. The results from this study showed that ELF-EMFs can induce apoptosis in osteocyte-like MLO-Y4 cells. In this study, the expression of the Bax gene was reduced and the expression of Bcl-2 was increased compared to the control group [39]. In another study, Hamed Reihani et al., examined the effect of a pulsed electromagnetic field (PEMF) on the intervertebral disc (IVD) in mice. The results of this study showed that apoptotic markers such as caspase 3 and Bax/Bcl-2 ratio were affected by the electromagnetic field, so regulating the expression of these apoptotic markers may be one of the mechanisms by which the electromagnetic field is effective in reducing disc resorption [40]. Also, Natalia Cichon et al., studied the effect of ELF-EMF on the molecular mechanism of apoptosis in post-stroke patients. They showed that ELF-EMF significantly increased the expression of BAX, CASP8, TNFα, and TP53 which are well-known pro-apoptotic genes that activate signaling pathways involved in brain plasticity processes in patients with post-stroke [41]. Differences in study conditions such as wavelength and frequency, wave intensity, exposure duration, and type of EMF generator are the main causes of contradictions between the results of different experiments.

According to the results, it can be concluded that extremely low-frequency electromagnetic field may lead to the PCD in MDA-MB-231 cells. In addition, changes in the expression level of apoptosis-related genes confirm this process. Thus, regulating the expression of apoptotic proteins may be one of the mechanisms by which ELF-EMF is effective in inducing programmed cell death in cancer. However, how an ELF-EMF induces cancer cells apoptosis is still vague. Nevertheless, the relationship between ELF-EMF and apoptosis in breast cancer cells and the underlying mechanism of such a process needs to be further studied. After further research, ELF-EMF therapy might be introduced as a new adjuvant approach that may contribute to anti-cancer research and further cancer therapy strategies.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICS APPROVAL

The project was found to be in accordance with the ethical principles and the national norms and standards for conducting medical research in Iran.

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