

# 1Hz 100mT Electromagnetic Field Induces Apoptosis in Breast Cancer Cells Through Up-Regulation of P38 and P21

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**Introduction:** Breast cancer is the most common cause of cancer-related death among women. Recently, extremely low-frequency electromagnetic field (ELF-EMF) has been proposed as a new interfering agent with future therapeutic potentials. Many studies have revealed that cellular processes such as apoptosis in breast cancer are affected by ELF-EMFs. However, more researches are needed to clarify the underlying mechanism of action for these fields. In this study, the apoptotic effect of ELF-EMF on the MC4L2 cell line was examined and the mRNA expression level of the P21 and P38 genes were further investigated.

**Methods:** A triple-positive mouse breast cancer cell line (MC4L2) was purchased from the Genetic Resource Center (Iran). This study was performed on two groups of ELF-EMF exposure (100mT/1 Hz for 5 days, 120 min each day) and sham exposure. Cell viability and apoptosis rate of both the exposure and sham exposure groups were determined by flow cytometry. Alterations in the P21 and P38 mRNAs expression levels were investigated; using real-time PCR.

**Results:** ELF-EMF exposure induced 30% apoptosis in MC4L2 cells compared with the control group. The mRNA expression level of P38 and P21 was significantly increased after ELF-EMF exposure compared to the control group.

**Conclusions:** ELF-EMF induces apoptosis in the MC4L2 triple-positive cell line. Furthermore, this exposure affects important gene expression involved in the cell cycle. Our data propose that ELF-EMF in a specific time, intensity and frequency could be beneficial for breast cancer treatment. However, more studies are required to confirm our findings.

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## INTRODUCTION

Breast cancer is the most common malignancy after lung cancer [1]. Several risk factors have been defined for this disease including

familial history, genetics pattern, estrogen and progesterone level, being overweight and obese, and alcohol consumption [2-4]. According to

Iran's statistical data, breast cancer starts at least one decade sooner in Iranian women compared with women in developed countries and appears in more advanced stages [5]. In addition, the disease is the second most common cause of cancer deaths [6]. By increasing the use of electrical devices, electromagnetic radiations with low frequencies have increased in our environment. In general, the electromagnetic and electric fields caused by different voltages and magnetic fields can be induced by electromagnetic waves [7]. The absorption and penetration of these waves depend on several factors including the frequency, the type of radiation, and the type of tissue that absorbs them [8]. Electromagnetic radiation is divided into two categories based on their effect on living organisms: The first group is the ionizing waves with direct and indirect biological effects which can damage the DNA molecule and genetic materials. The second group is non-ionized waves which include low-frequency waves, long wavelengths, and low penetration powers which apparently do not have enough energy to break the chemical bonds of molecules and atoms [8, 9]. Several studies have shown the relation between the electromagnetic field (EMF) exposure and health effects, e.g. the potential association between the increased exposure to high-frequency magnetic fields (MF) and increased risk of various diseases including childhood and adult leukemia. However, the conclusions of these studies are sometimes difficult to interpret and are therefore controversial [10-13]. Amounting studies have revealed that ELF-EMF can affect different cellular processes such as apoptosis and proliferation which are involved in cancer development [14-20]. There is a plausible hypothesis of the way ELF-EMF impacts cellular behavior. One of these hypotheses could be the effect of ELF-EMF on membrane structure and permeability to small molecules [21-23]. Therefore, with regard to the previously obtained results, we conducted this study to investigate the alterations in the expression level of important genes involved in cell cycle after exposure to the very low-frequency field over a given period of time. Additionally, we studied the impact of this field on apoptosis induction in a triple-positive breast cancer cell line.

## **METHODS**

### **ELF-EMF Exposure System and MC4L2 Cell Culture**

MC4L2 mouse breast cancer cell line was purchased from the Genetic Resource Center (Iran). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco); supplemented with 10% fetal bovine serum and 1% antibiotic solution (Gibco). Cells were cultured in standard conditions (37°; 5% CO<sub>2</sub>). The culture medium was changed every three days. When the confluency of cultured cells reached to 80%-90%, they were washed with phosphate-buffered saline (PBS), detached by 0.25% trypsin (Gibco, USA), and subcultured in 35 mm<sup>2</sup> culture dishes with a density of  $5 \times 10^4$  cells/well. For ELF-EMF exposure, cells were exposed to ELF-EMF (Parto Farazane Farda, Iran) with the frequency of 1 Hz, and 100 mT intensity, for 5 days, 2 hours each day. Sham exposure was utilized as the control of this study (the same condition as the experimental group just without the ELF-EMF exposure).

### **QRT-PCR**

The total RNA was isolated from cells; using the RNX total RNA isolation kit (SinaClon, Iran) according to the manufacturer's protocol. One  $\mu$ g of RNA was reverse transcribed into cDNA; using a high fidelity superscript, reverse transcriptase kit Vivantis (Vivantis, Malaysia) according to the manufacturer's instructions. The mRNA expression level of the following genes was analyzed by real-time PCR performed on an ABI 7500 (Applied Biosystem, USA), and  $2^{-\Delta\Delta C_t}$  method [24-26]. Optimal oligonucleotide primers used in the above real-time PCR were designed by gene runner software and synthesized by SinaClon (Iran) for Mus musculus mRNAs sequences: GAPDH (internal control), 5'-GTCGGTGTGAACGGATTTGG-3' (sense) 5'-GCCTTGACTGTGCCGTTGAAA-3' (antisense); P21, 5'-CAGCAGAATAAAAGGTGCCACA-3' (sense), 5'-CATGAGCGCATCGCAATCAC-3' (antisense); P38, 5'-GCATCATGGCTGAGCTGTTG-3' (sense) and, 5'-CTGGGGTTCCAACGAGTCTT-3' (antisense).

### **Flow Cytometry Analysis of Apoptosis**

MC4L2 was exposed to 100 mT, 1Hz ELF-EMF

2hours/day for 5 days and the sham exposure group were studied for apoptosis induction by Annexin V/PI flow cytometry. Briefly, cultured cells ( $5 \times 10^5$  cells) were washed with PBS, centrifuged for 5 minutes at 1200 RPM, and incubated in 500  $\mu$ l binding buffer 1X containing 5  $\mu$ l Annexin V-FITC and 3  $\mu$ l Propidium Iodide in the dark for 15 min at 4°C temperature. Then, the stained samples were analyzed by the flow cytometer (BD FACS Calibur, USA).

**Statistical Analysis**

All statistical analyses in this study were performed by Graphpad Prism software version 6.01. To compare different groups with each other t-test was performed and p values less than 0.05 were considered significant. All experiments were performed in duplicate and repeated at least 2 times.

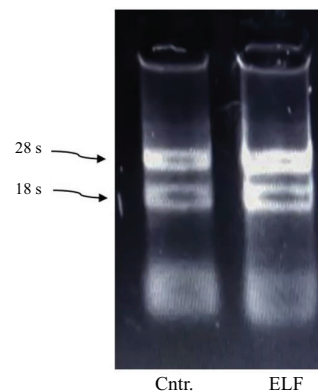
**RESULTS**

Results obtained from flow cytometry:

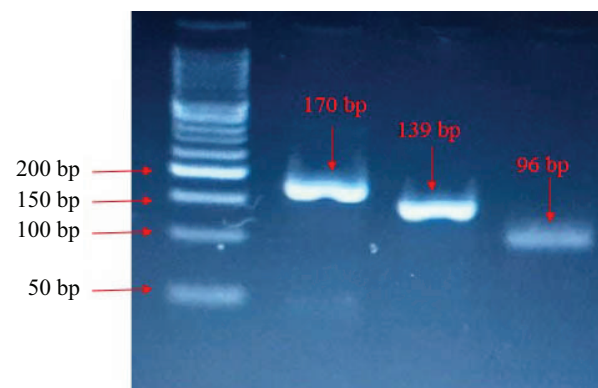
According to the results of flow cytometry graphs, about 15% of cells in the sham exposure control group had early + late apoptosis. This rate was about 45% in the experimental group (exposed to the EMF) (Figure 1).

**Real-Time PCR**

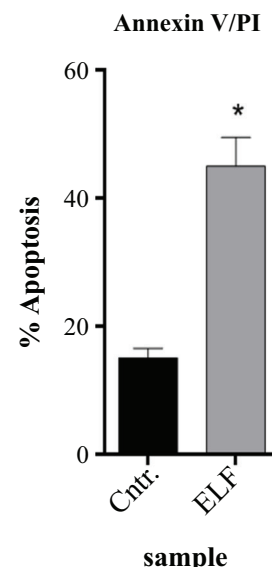
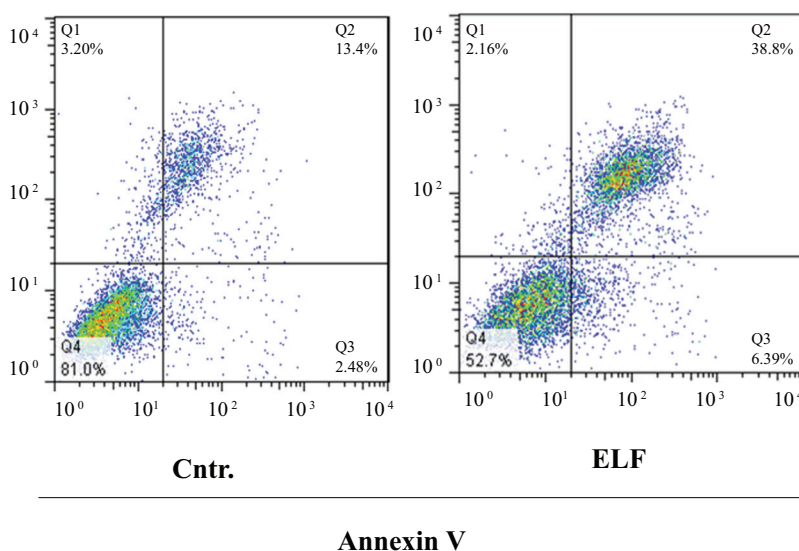
The quality of extracted RNAs was investigated on



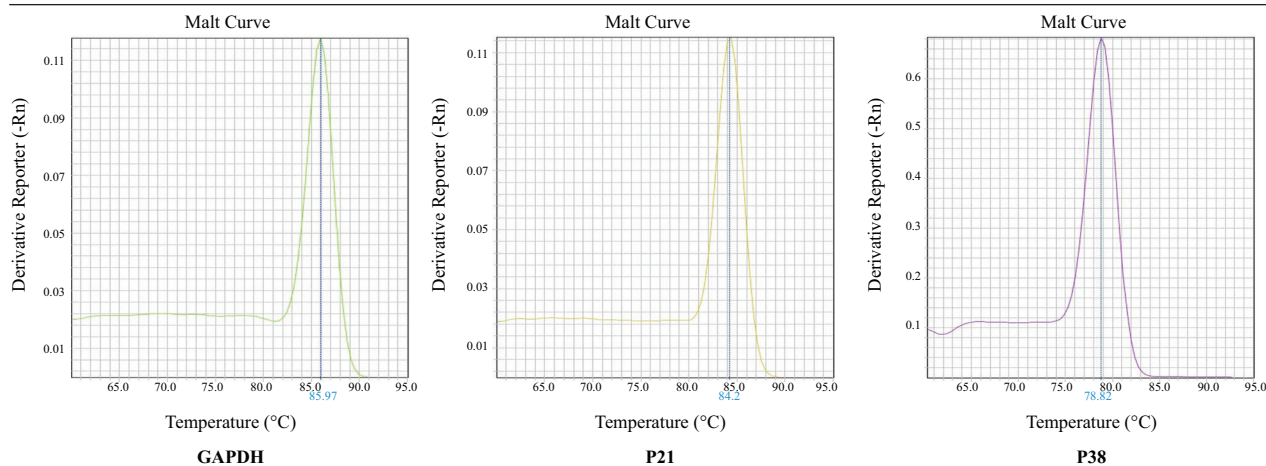
**Figure 2:** Electrophoresis of Extracted RNAs for Control and Experimental Samples



**Figure 3:** Electrophoresis of RT-PCR Product for GAPDH (as the Internal Control), P21, P38



**Figure 1:** Flow Cytometry Data of ELF-EMF Exposure and Sham Exposure  
 Q4: Population of live cells (AnnexinV - /PI -), Q3: population of early apoptotic cells (AnnexinV + /PI -), Q1: population of necrotic cells (AnnexinV - /PI +), Q2: population of late apoptotic cells (AnnexinV + /PI +).



**Figure 4:** Graph Charts of the Melt Curve for GAPDH, P21, and P38 Genes Products

a 1% agarose gel electrophoresis (Figure 2). These RNAs were then employed for cDNA synthesis and subsequent real-time PCR. According to the results of the real-time PCR, P21 and P38 mRNAs expression levels were increased in the samples exposed to the ELF-EMF compared to the sham exposure (Figure 3).

**Investigating Specific Amplification**

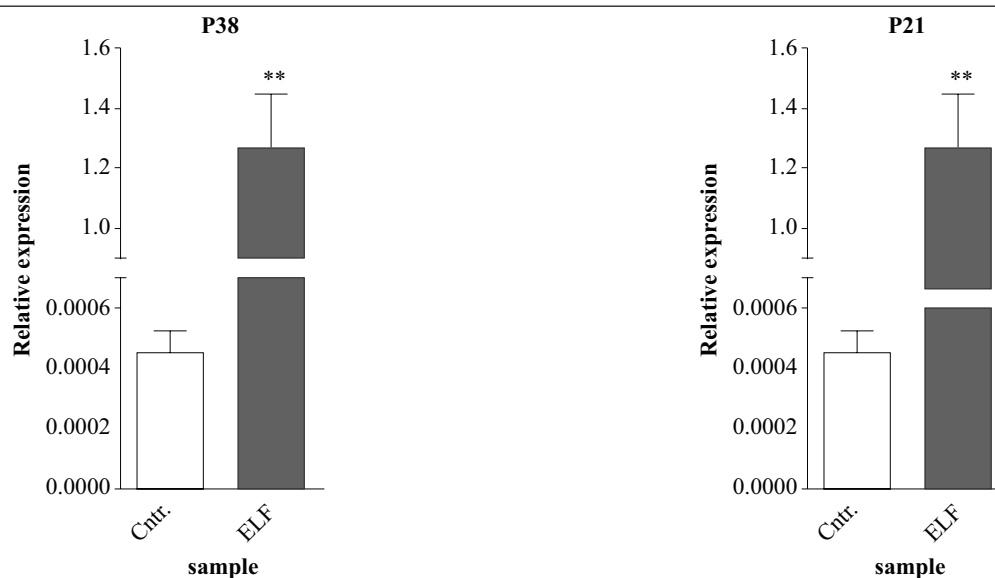
The melt curve analysis revealed specific amplification of target products (Figure 4). To further investigate specific amplification, after real-time PCR, the products were electrophoresed on 2% agarose gel. Based on designed primers the amplicon lengths were intended to be 170 bp, 139 bp, and 96 bp for GAPDH, P21, and P38 genes, respectively (Figure 3).

**DISCUSSION**

According to statistics, cancer is one of the leading causes of death worldwide. Recently, EMFs, known to be modulators of proliferation rate, enhancers of apoptosis, and inductors of genotoxicity seems to provide a new emerging approach for cancer treatment. It is demonstrated that ELF-EMF can affects important cellular processes including apoptosis in breast cancer cells [27]. However, further researches are needed to find out the possible mechanisms behind this process. In our study, MC4L2 cell line was studied in two groups of experimental and sham exposure. Viability and/or apoptosis rate of the exposure (100mT/1 Hz for 5 days, 120 min each day) and sham exposure groups were determined by flow cytometry. Alterations at the P21 and P38 mRNA

expression levels were investigated to further elucidate the ELF-EMF radiation on the important genes in the cell cycle process. Amounting studies have demonstrated that EMFs can affect important cellular processes including apoptosis in cancer cells. However, there is relatively little consensus on the biological effects of EMFs. This is in part due to the diversity in EMF characteristics applied in different experiments and somehow to the complexity in cellular responses to EMFs [28-30]. In this study, we exposed cells to 100mT and 1Hz ELF-EMF for 5 days, 2h/day. Our results revealed that in the mentioned characteristics of the field, the rate of apoptosis increased to about 30% in the exposed cells compared with the sham exposure.

A 100-mT EMF was utilized in the study of Tatarov et al., on the Eph4-MEKbcl2 cell line with a frequency of 1 Hz for 360 minutes a day for 4 weeks to investigate tumor growth and tumor necrosis; using the in vivo imaging system [31]. They reported a significant reduction in tumor growth in in vivo condition. In the present study, MC4L2 breast cancer cells were used to examine the effect of ELF-EMF on apoptosis as well as the P21 and P38 gene expression. Similarly, the results of our study demonstrated that ELF-EMF (100mT/1 Hz for 5 days, 120 min each day) causes in vitro apoptosis in the mentioned cell line (Figure 1). In 2007, Aldinucci also observed increased induction of apoptosis in human cancer cells after exposure to radiation of 50 Hz EMF magnetic field of 1 mT [32]. Similar results were obtained in a study on the MCF-7 cell line and the normal MCF-10 cell line at 20 Hz; using an EMF of 2,3,5 mT for 60 minutes



**Figure 5:** Real-Time PCR Data for Different Groups of ELF-EMF Exposure (ELF) and Sham Exposure (Ctr.)

After exposure, P38 and P21 mRNA expression levels increased significantly compared with the sham exposure ( $P=0.0088$  and  $0.0063$ , respectively).

a period of 3 days. Cytotoxic effects on MCF-7 cell line have been observed and no changes have been made on the normal MCF-10 cell line [33]. Other experiments have shown that human lymphoblastic cells that were exposed to 0.4 to 1 mT/60 Hz EMF and dexamethasone simultaneously, had an increase in programmed cell death; while exposure to a frequency of 50 Hz with a magnetic field of 2 mT has little effect on the process [34]. It seems that the differences in study conditions such as wavelength and frequency, the intensity of wave type, radiation duration, and magnetic field generator type is the main contradiction between the results of various experiments. Some researchers give different explanation for this discrepancy, it seems that cutting off the brain and other organs, and preparing slides can be considered as the main reason for various responses [35-38]. P21 and P38 are some of the most important signaling pathways activated by different stress factors; resulting in apoptosis [39]. P38 regulates the production of inflammatory mediators and controls reproduction, differentiation, migration, and survival of cells. Activating them in endothelial cells leads to actin recovery, angiogenesis, and response to DNA damage [40]. P21 can inhibit the proliferation of cells independently of inhibiting the proliferating cell nuclear antigen (PCNA) that is required to progress the S phase. Some anti-proliferative P21 activities depend on protein metabolism or multiple

proteins that act in transcriptional regulation of genes [41]. In 2010, Kim et.al reported that activation of mapk-P38 signaling pathway is effective in inducing programmed cell death. It was reported that exposure of cancer cells and fibroblast cells to a 60 Hz magnetic field at 6 ms for 30 min every 24 hours over a duration of 3 days did not alter the total P38 expression; however, its expression level alteration was observed after 48 to 72 h. The P38 phosphorylation rate increases as the cell viability decrease. These results suggest that repeated low-frequency magnetic field exposure may lead to activation of mapk-P38 signaling pathway and induction of programmed cell death [39]. In our study, P21 and P38 gene expression were examined in both the experimental and sham exposure groups. Both genes showed up-regulation at mRNA level after exposure compared with the control group (P38 and P21 mRNA expression level were about 2700 and 2000 fold more than that of the sham exposure, respectively) (Figure 5). According to the results, we can conclude that the mentioned field induces programmed cell deaths in the MC4L2 cells. Moreover, increasing the mRNA expression levels of P21 and P38 genes after ELF-EMF exposure, proposes a possible particular pathway for this effect. However, the association between ELF-EMF and apoptosis in breast cancer cells and the underlying mechanism of such action requires further studies.

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## CONFLICT OF INTEREST

The authors declared no conflict of interest.

## ETHICS APPROVAL

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