# Methotrexate cytotoxicity on MCF-7 breast cancer cells is not altered by exposure to 25 Hz, 1.5 mT magnetic field and iron (III) chloride hexahydrate

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## Abstract

The action of electromagnetic fields (EMF) on different pathways related to cell physiology, proliferation, toxicity of chemicals, gene expression, etc., are currently being investigated although the results are still not conclusive and even conflicting. In laboratory and animal studies, EMF has been found to produce a great variety of effects such as: increase in ornithine decarboxylase activity in breast, increase in $\beta$-galactosidase gene expression and oncogene transcription after exposure to 50/60 Hz. Animal studies have shown that the use of EMF can enhance drug delivery across biological barriers (rat abdominal skin), using benzoic acid as the drug candidate. It has been reported by different authors that pulsed EMF (PEMF) can produce alterations in antineoplastic drugs potency. In the present study, we investigated the effects of PEMF on methotrexate cytotoxicity in MCF-7 breast cancer cells and the effects with simultaneous exposure to $FeCl_3$. The data presented in the current report indicate that PEMF (25 Hz, 1.5 mT) do not induce modulation of the action of methotrexate (with and without iron-III) in MCF-7 cells when they are exposed to PEMF for 2 h/day during 3 days.
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*Keywords:* Pulsed electromagnetic fields (PEMF); Drug resistance; Methotrexate; MCF-7; Human breast cancer cells; Iron ion

## 1. Introduction

Electromagnetic fields (EMF) have become in public interest due to a possible association with cancer risk, promotion of established cancer or other undesirable biological effects. The action of EMF on different pathways related to cell physiology, proliferation, toxicity of chemicals, gene expression, etc., are currently being investigated although the results are still not conclusive and even conflicting.

In laboratory and animal studies, EMF has been found to produce a great variety of effects such as: increase in ornithine decarboxylase activity in breast [1], increase in $\beta$-galactosidase gene expression [2] and oncogene transcription after exposure to 50/60 Hz [3]. In this way, it has been reported by Grissom [4] that EMF of moderate amplitudes (1–100 mT) may produce alterations in different enzyme activities. A broad spectrum of interaction mechanisms can occur between power frequency EMF and living organisms. The best known is the production of currents by magnetic induction [5].

Trace metal ions like iron plays important roles as binding, transport and storage of molecular dioxygen in a wide variety of living systems and in general terms most ions can bind to proteins, peptides, amino acids, DNA, sugar and lipids [6]. It is well known that static magnetic fields affect the diffusion of biological particles in solutions through the Lorentz force and Maxwell stress [7]. Hilger et al. [8] found that iron-containing molecules such as iron oxide, magnetite, are able to induce considerable heating effects in the surroundings. Other authors have reported that only static EMF has an inhibitory effect on iron-induced lipid peroxidation [9]. However, the effects of non-static EMF and pulsed EMF (PEMF) should be more

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investigated. In this way, Zmyślony et al. [5] have found that only the simultaneously exposure of lymphocytes to $FeCl_2$ and 7 mT EMF produce an increase in DNA damage, reaching about 20% for static EMF and 15% for 50 Hz EMF. The studies have lead to conflicting results, but now a new controversy has appeared: the simultaneous exposure of cells to EMF or to PEMF and Fe ions could produce cellular alterations.

Breast cancer is one of the leading forms of cancers in women. Epidemiological data suggest that exposure to power frequency (50/60 Hz) EMF may be a risk factor for breast cancer in humans [10]. If exposure to EMF contributes to the etiology of breast cancer, it is likely that they must stimulate the growth of breast cells, damage DNA or enhance the effects of chemicals or antineoplastic drugs [11].

The resistance of tumour cells to different antineoplastic agent is an obstacle for cancer chemotherapy. The main mechanism in drug resistance is the multidrug resistance (MDR) phenomenon, which constitutes the reduction of intracellular drug level due to the P-glycoprotein pump function [12]. Animal studies have shown that the use of EMF can enhance drug delivery across biological barriers (rat abdominal skin), using benzoic acid as the drug candidate [13]. It has been reported by various authors that PEMF can produce alterations in antineoplastic drugs cytotoxicity [14–17]. In addition, the use of unpulsed magnetic fields has been found to produce alterations in MCF-7 cells. In this way, Harland and Liburdy [18] have observed that 1.2 μT, 60 Hz EMF partially blocked tamoxifen's inhibitory action on the growth of this human mammary tumour cells in vitro.

So far, there have been very few studies on tumour cells, which investigate the potential effects of PEMF with frequencies below 50/60 Hz. In the present study, we investigated the effects of 25 Hz, 1.5 mT PEMF on methotrexate cytotoxicity in MCF-7 breast cancer cells as well as the effects with simultaneous exposure to $FeCl_3$.

## 2. Materials and methods

### 2.1. Cell culture

MCF-7 cells, a human breast cancer cell line, were cultured in Dulbecco's modified Eagle's medium nutrient mixture F12-HAM (DME/F12-HAM) (with L-glutamine and HEPES), supplemented with sodium bicarbonate 7.5% (28 ml/l), 5% foetal bovine serum and 1% antibiotic–antimycotic solution (100 ×) (PSF, Gibco) at 37 °C in a 5% $CO_2$/air atmosphere. These cells grow in monolayer and were subcultured with trypsin (0.05%) and EDTA (0.02%) in Dulbecco's phosphate-buffered saline (PBS).

The antineoplastic drug used was methotrexate (Lederle-Cyaramid Ibérica, Madrid), a potent inhibitor of dihydrofo-

late reductase. The iron-containing molecule used was iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) (Merck). Stocks were prepared in sterile PBS and frozen at $-20$ °C until they were used.

### 2.2. Magnetic field exposure system and current density

The equipment used (Pulsatrón, CEM-84/J; J&J Electromédica; Málaga, Spain) generates rectangular voltage pulses (25 Hz) that feeds two air core coils of $15 \times 10.5$ cm (Helmholtz type), used for the exposure. The frequency used in this study is the maximum obtainable value with this equipment. The generated peak magnetic field between coils was 1.5 mT peak. The general characteristics of the voltage waveform applied to the coils, the electric field and the induced electric current density were reported in a previous work [19].

Briefly, the voltage waveform applied to the coils produced groups of 15 rectangular pulses, repeated at 25 Hz. The pulses were positive in intervals corresponding to 180 μs when voltage was applied and negative in 20-μs gaps. The effective electric field value in each culture well fluctuated from 65 to 260 mV/m during the positive pulse (corresponding to the 180 μs portion) and from 650 to 1600 mV/m in the negative pulse zone (corresponding to the 20 μs between rectangular pulses), depending on the spatial location of each well. The calculated values of induced electric current density fluctuated from 97.5 to 390 mA/m$^2$ in the positive pulse period and from 975 to 2340 mA/m$^2$ in the negative pulse period.

### 2.3. Cells exposure protocol

Cells were cultured in 24-well dishes and incubated, during the PEMF exposure, at 37 °C inside a commercial cell culture incubator made of plastic (Thermocult, Boehringer-Mannheim, Germany). The magnetic field distribution ran perpendicular to the cell culture surface (Fig. 1).
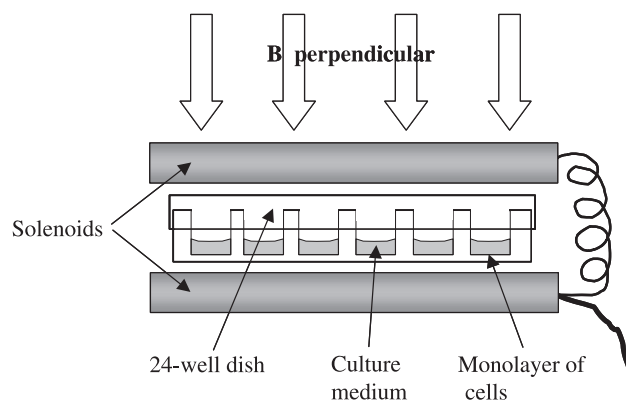


Fig. 1. Magnetic field exposure system. The magnetic field distribution ran perpendicular to the cell culture surface. The cells were cultured in 24-well dishes, in a monolayer on the bottom of the wells.

The control cells were placed with no current running through the coils, immediately after the PEMF exposed cells. They were exposed in the same incubator, although not at the same time. Therefore, there was no added artificial magnetic field at the control location. The same passage cells were simultaneously used for their matched sham and exposed experimental groups [20]. There was no additional heating due to the activation of the coils, as measured directly by a conventional thermometer.

After monolayer trypsinization, 5000 exponentially growing cells were seeded in each well and were incubated for 24 h at 37 °C, 5% $CO_2$, to allow the cells to attach. Then, the experiments were performed as follows:

(a) continuous exposure to methotrexate, and exposure to PEMF for 2 h/day for 3 days.
(b) continuous exposure to methotrexate and $FeCl_3 \cdot 6H_2O$, and exposure to PEMF for 2 h/day for 3 days.

Next, the cells were incubated for an additional time of 24 h at 37 °C, 5% $CO_2$. As soon as the incubation period was over, viability was measured via the neutral red stain cytotoxicity test [21,22].

The methotrexate doses assayed ranged from 0.01 to 70.0 μg/ml. Iron (III) dose used was 10 μg/ml. This dose was the higher one obtained from the cytotoxicity curves for $FeCl_3 \cdot 6H_2O$, which did not show decrease in surviving fraction (Fig. 2).



Fig. 3. Effect of 25 Hz, 1.5 mT pulsed electromagnetic field (2 h/day, for 3 days) on MCF-7 breast cancer cells exposed to methotrexate. Mean ± S.D. of three independent experiments in triplicate. PEMF: pulsed electromagnetic field. $p > 0.05$ Student's $t$-test.

### 2.4. Iron (III) chloride hexahydrate cytotoxicity

$FeCl_3 \cdot 6H_2O$ cytotoxicity was assayed by exposure of MCF-7 cells to different doses and surviving fraction at each dose was used to make dose–response curves with and without PEMF exposure.

A total of 5000 exponentially growing cells were seeded in each well in a 24-well dish and then incubated for 24 h at 37 °C, 5% $CO_2$. Then, the cells were exposed to different doses of $FeCl_3 \cdot 6H_2O$ (0.0025–900 μg/ml) in 72 h at 37 °C, 5% $CO_2$, and after this period, viability was measured by the neutral red stain cytotoxicity test. The exposure to PEMF was performed during 2 h/day for 3 days.

### 2.5. In vitro cytotoxicity assay

The cytotoxicity test with neutral red stain [22] was applied to measure cell viability. This is a test based on the lysosomal adding of supravital neutral red stain, which quantifies the number of viable cells after exposure to a physical or chemical agent. The quantification of the stain extracted from cultured cells has been shown to be linear with the number of viable cells through direct count [21]. Although this test does not allow conclusions concerning toxicity mechanisms, it is possible to evaluate the number of viable cells after a certain period in a similar way as other cytotoxicity tests, allowing operators to calculate the surviving fraction of cells after a specific physical or chemical treatment.

For this assay, the culture medium was replaced by 1 ml of supplemented fresh medium with 40 μg/ml neutral red. The new medium had been previously incubated 24 h at 37 °C and centrifuged at 2500 rpm for 10 min to avoid crystalline stain precipitates. After 3 h of plate incubation in the presence of neutral red, the medium
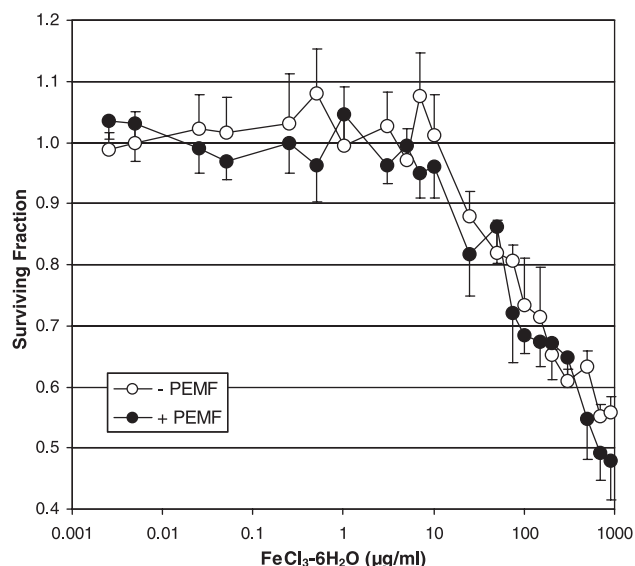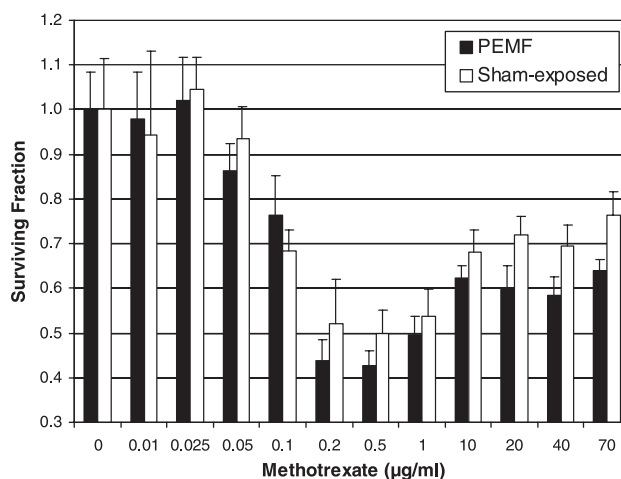


Fig. 2. Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) cytotoxicity curves obtained for MCF-7 breast cancer cells with and without pulsed electromagnetic field exposure (2 h/day, for 3 days). Iron (III) dose used, to study the combined effects with methotrexate and pulsed electromagnetic field, was 10 μg/ml. This is the higher dose obtained from the curves, which did not show decrease in surviving fraction. Mean ± S.D. of three independent experiments in triplicate. − PEMF: without pulsed electromagnetic field; + PEMF: with pulsed electromagnetic field.

was removed and cells were washed in 1 ml of fixative (1% $CaCl_2$/0.5% formaldehyde). Next, 1 ml of 1% acetic acid/50% ethanol solution was added to each well to extract the stain [23]. After 10 min at room temperature and subsequent shaking, the optical density (OD) was measured at 540 nm. The mean OD in the control cells without drug determined after incubation was regarded as 100%, and the percentage of survival at each drug dose was calculated.

### 2.6. Statistical analyses

The Wilk–Shapiro rankit-plot test was used to assess the normal distribution of the data. Additional statistical analyses were made with the Student's *t*-test. Differences were considered significant when $p < 0.05$.

## 3. Results

### 3.1. Iron (III) chloride hexahydrate cytotoxicity

Fig. 2 shows the cytotoxicity curves obtained for $FeCl_3 \cdot 6H_2O$ on MCF-7 breast cancer cell line with and without PEMF exposure. The doses assayed ranged from 0.0025 to 900 μg/ml. The higher iron (III) dose, which did not show decrease in surviving fraction, was 10 μg/ml. Subsequently, this was the dose used to study the combined effects with methotrexate and PEMF.

### 3.2. Effect of PEMF on methotrexate cytotoxicity

As shown in Fig. 3, the 25-Hz, 1.5-mT PEMF exposure did not induce alterations on methotrexate cytotoxicity in relation to sham-exposed control cells, as
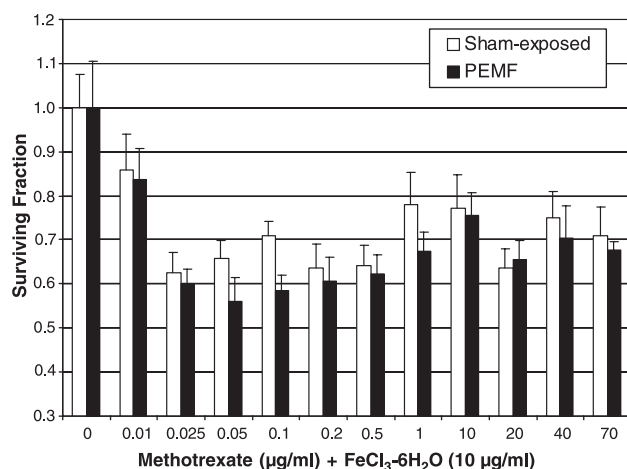
Fig. 4. Effect of 25 Hz, 1.5 mT pulsed electromagnetic field (2 h/day, for 3 days) on MCF-7 breast cancer cells exposed to methotrexate and iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$). Mean ± S.D. of three independent experiments in triplicate. PEMF: pulsed electromagnetic field. $p > 0.05$ Student's *t*-test.

measured via the neutral red cytotoxicity assay; $p > 0.05$ Student's *t*-test. Methotrexate doses assayed ranged from 0.01 to 70 μg/ml.

### 3.3. Effect of PEMF on methotrexate cytotoxicity under exposure to iron (III) chloride hexahydrate

The higher dose of $FeCl_3 \cdot 6H_2O$ (10 μg/ml), obtained from the dose–response curves (Fig. 2), that did not show cytotoxic effect was used to expose MCF-7 cells to methotrexate and PEMF. No changes were observed in methotrexate potency after exposure to PEMF in the presence of iron (III), compared to sham-exposed controls cells, $p > 0.05$ Student's *t*-test (Fig. 4). Nevertheless, the presence of iron (III) (10 μg/ml) produced an increment of 40.1% and 29.4% in methotrexate potency at 0.025 and 0.05 μg/ml, respectively; with $p$ value of < 0.01, Student's *t*-test. Iron (III) did not induce alterations in methotrexate cytotoxicity at the other drug doses assayed.

## 4. Discussion

In recent years, numerous papers concerning the biological effects of pulsed electromagnetic fields (PEMF) have been published.

Hypotheses explaining the influence of electromagnetic fields (EMF) on living systems have been widely proposed. Most of them share the common assumption that the cell membrane is the target for the primary field interaction, thus influencing the signal transduction mechanisms and affecting several biological endpoints [24].

Methotrexate is a potent inhibitor of dihydropholate reductase that has been used as effective antineoplastic treatment for breast cancer due to its capacity to inhibit cell growth [25]. It is well known that only trace amounts of iron remain free as non-chelated or loosely chelated iron by the action of different Fe-binding proteins in vivo. In our experiments, this situation was reduced to a minimum due to the in vitro experimental conditions where only the 5% of the culture medium correspond to foetal bovine serum. Therefore, the importance of the complexation rate of $FeCl_3$ with the different proteins of the culture medium could be considered insignificant. Both iron ions, ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$), may participate in oxidative reactions, triggering oxidative damage to cellular components, being the Fenton reaction ($Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + \cdot OH + H_2O$), a well-known phenomenon to induce oxidative stress. $Fe^{3+}$ participates indirectly in this reaction, when reduced to $Fe^{2+}$. $Fe^{3+}$ alone directly stimulates lipid peroxidation, because of the accompanying generation of superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide ($H_2O_2$) and the hydroxyl radical ($\cdot OH$) [26].

In the present study, we evaluated the effects of PEMF on methotrexate cytotoxicity in MCF-7 cells. We observed no significant alterations in surviving fraction when cells were

exposed to different doses of methotrexate and PEMF (25 Hz, 1.5 mT). Besides, no effects were observed when MCF-7 cells were co-treated with $FeCl_3 \cdot 6H_2O$, methotrexate and PEMF. A decrease in toxicity was observed as methotrexate concentration was increased to over 10 μg/ml. This finding could be explained by different molecular mechanisms such as saturation or competitive processes, which require further studies.

A previous report indicates that PEMF exposure (25 Hz, 1.5 mT) can produce alterations in the cytostatic action of mytomicin C and cisplatin in a human colon adenocarcinoma cell line (HCA-2/1$^{cch}$) [19].

Our findings are in disagreement with a number of articles that show the effects of different field exposures on the uptake and potency of different drugs. In this way, Omote [27] and Omote et al. [14] reported that PEMF increases the cell uptake of $^3$H-methotrexate. They suggest that PEMF promotes the uptake of antitumor agents by cells. In the same way, Liang et al. [16] demonstrated that PEMF enhanced the potency of daunorubicin against the KB-ChR-8-5-11 subline when this drug is added prior to PEMF exposure. In addition, Hannan et al. [15], Harland and Liburdy [18] and Blackman et al. [28] found an inhibition of the antiproliferative action of tamoxifen and melatonin in MCF-7 cells, and Salvatore et al. [29] reported that EMF improved the neoplastic cell killing by antineoplastic chemotherapy.

There is no previous report of studies concerning the effects of EMF on the antineoplastic activity of drugs simultaneously exposed with iron containing molecules. Nevertheless, some authors have studied the effects of EMF on cells exposed only to $FeCl_2$, $FeCl_3$ and other metals.

In this way, it has been recently reported that EMF (7 mT static and 50 Hz) can induce DNA damage when lymphocytes are simultaneously exposed to $FeCl_2$ at non-cytotoxic concentrations (10 μg/ml) [5]. These authors hypothesise that the number of reactive oxygen species generated by iron cations may substantially increase. In an attempt to determine whether EMF exposure might lead to DNA damage, Lourencini da Silva et al. [30] exposed the pBR322 plasmid to EMF in the presence of a transition metal ($SnCl_2$), obtaining positive correlation between exposure to EMF and DNA damage. These authors affirm that their observations support the idea that EMF probably through secondary generation of reactive oxygen species can be clastogenic.

In contrast, other authors have found that only static magnetic fields have an inhibitory effect on iron-induced lipid peroxidation and that the effectiveness of this magnetic field on iron ion-induced active oxygen species generation is restricted to a so-called "window" of field intensity of 2–4 mT [9].

Our results are in disagreement with the findings reported by other authors and with the previous work carried out in our laboratory using the same PEMF but different cells (human colon adenocarcinoma) and drugs (mytomicin C and cisplatin) [19].

These contradictory results suggest that appropriate drug, dose, cell line and/or treatment schedules could be important factors for PEMF modulation of cytotoxicity.

On the other hand, a statistically significant increment of 40.1% and 29.4% in methotrexate potency was observed when cells were exposed simultaneously to 10 μg/ml of iron (III) and methotrexate at 0.025 and 0.05 μg/ml, respectively. This finding has to be treated with care; more studies are required to clarify the role of $FeCl_3 \cdot 6H_2O$ as a possible chemosensitizing agent or enhancer of methotrexate cytotoxicity.

The data presented in the current report indicate that PEMF (25 Hz, 1.5 mT) does not induce modulation of the action of methotrexate (with and without iron-III) in MCF-7 cells when they are exposed to PEMF for 2 h/day during 3 days.

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