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Cytotoxicity of carboplatin on human glioblastoma cells is reduced by the concomitant exposure to an extremely low-frequency electromagnetic field (50 Hz, 70 G)

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ABSTRACT

Glioblastoma multiforme (GBM) is a malignant brain cancer that causes high mortality in patients. GBM responds weakly to the common cancer treatments such as chemotherapy and radiotherapy and even surgery. Carboplatin is an alkylating agent widely used to treat cancer. However, resistance to this drug is a common problem in its use in cancer treatment. Concomitant exposure to extremely low-frequency electromagnetic fields (ELF-EMFs) and carboplatin is one unexplored possibility for overcoming this resistance. Indeed, many lines of evidence show that EMF affects cancer cells and drug action. In this study, we evaluated the effect of concomitant administration of carboplatin and EMF (50 Hz, 70 G) and also concomitant administration of carboplatin and static magnetic field (SMF) (70 G) on human glioma cell line (U-87). The results showed that cotreatment reduced the efficiency of carboplatin in U-87 cells, by decreasing caspase-3 in comparison to drug groups. Overall, EMF reduced the apoptotic effect of carboplatin, possibly through a redox regulation mechanism. Therefore, we have to avoid coadministration of magnetic field (MF) and carboplatin in tumor area, because the MF decreased the toxicity of the drug. However, further studies are needed to reveal the action mechanism of this combination therapeutic method.

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Glioblastoma multiforme (GBM); human glioblastoma cell (U-87); carboplatin; electromagnetic field (EMF); apoptosis

Introduction

In the recent years, much debate has been raised concerning the possible health effects of magnetic fields (MFs) on the human brain cancer. In this regard, clinical and epidemiologic data have indicated different effects (Bell et al., 1992; Mann & Roschke, 1996).

Glioblastoma Multiforme (GBM) is the most common and most malignant type of glioma, as it has a mean survival time of 14.6 months. Various therapeutic strategies including surgery, radiotherapy and chemotherapy are conducted to treat GBM. There are several genetic alterations associated with GBM formation and progression such as mutations, amplifications, epigenetic and gene expression changes (Beroukhi et al., 2007; Futreal et al., 2004; Lokker et al., 2002).

Living organisms are continuously exposed to the natural geomagnetic field of around 20–70 μ T.

The frequency of exposure to electromagnetic field (EMF) has increased with rapid advances in science and technologies such as magnetic resonance imaging (MRI) diagnosis, nuclear magnetic resonance (NMR) spectroscopy and passenger transport systems, which are based on magnetic levitation (Saunders, 2005).

Epidemiologic data have indicated that not all types of tumors are sensitive to EMFs. In this connection, the incidence of some specific types of leukemia and nerve cell tumors (especially from glial cells) increase severely with exposure to EMFs (Floderus et al., 1993).

Magnetotherapy provides a noninvasive approach to treat damages and inflammation and inhibit tumor growth. On the other hand, EMF influences the biological system by increasing the production of reactive oxygen species (ROS) that leads to breaking of DNA strands and cell death. In the recent years, free radicals released on malignant tumors under EMF 50 Hz, 1 mT

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and 0.2 T exposure have been a subject of intense research (Gurney & Van Wijngaarden, 1999).

Chemotherapy is another treatment for dealing with cancer. Carboplatin is a cell cycle nonspecific drug that modifies the DNA structure and inhibits DNA replication, thereby causing cell death. This alkylating agent is used in the treatment of malignant gliomas. Carboplatin and its analog cisplatin have their own merits and demerits when used as chemotherapeutic agents. In this regard, carboplatin (≈ 500 $\mu\text{g/ml}$) shows good activity against brain tumor cell lines (Haley et al., 2014; Rose et al., 2003; Vaughn, 2000).

The aim of the current study is to investigate the effects of MF (static and frequency) at frequencies 50 Hz and intensity 70 G on the GBM cell line, U-87 in the presence of carboplatin, an anticancer medication.

Materials and methods

Cell culture and reagents

The human glioblastoma U-87 cell line (pasture Institute, Tehran, Iran) was cultured in 25 cm^2 flasks (Iwaki, Tokyo, Japan) at a cell density of 12,000 cells/ml in Dulbecco's Modified Eagle's Medium (DMEM, Product Code: 11966025 Invitrogen Inc. Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% heat inactivated, sterile-filtered, fetal calf serum (Product Code: 12306C –200 ml Sigma-Aldrich Co., St. Louis MO, USA), and 1% penicillin–streptomycin solution (Product Code: P4333-100 ml, Sigma-Aldrich Co.) in a humidified atmosphere of 5% CO_2 at $37 \pm 0.5^\circ\text{C}$. The medium was replaced every 2 d. Cultures were routinely trypsinized at about 80% confluence and split with a ratio of 1:3 (Product Code of Trypsin: T4549-

100 ml, Sigma-Aldrich Co.). The cells were cultured under the same conditions using 96-well plates or 25 cm^2 flasks (Iwaki) to exclude any influence of the different cell containers. Then, the cells were simultaneously treated with carboplatin and EMF (static and frequency).

A 405 mM stock solution of carboplatin was obtained from Red Crescent Pharmacy, Kerman. Stock solutions were diluted in a culture medium immediately before use. Carboplatin (2.7 mM) was diluted and added to cells that were growing on cell culture dishes. All assays were performed after 24 h of incubation.

Exposure to static magnetic field (SMF) and extremely low frequency EMF

SMF was produced by three magnetic parallelepipeds of NdFeB, sized 50, 50 and 24 mm, coated with Ni, grade N40, Br 1260–1290 mT, magnetized through the thickness and supplied by Webcraft GmbH (Uster, Switzerland).

The magnets were kept together by attraction force in a structure made of six shelves of Plexiglas. The shelves of Plexiglas (thickness of 4 mm) were fixed using four bolts (8 mm of diameter) of polyvinyl chloride (PVC) (Figure 1a). These materials did not disturb the MF configuration. Magnetic inductions were measured using a Gaussmeter GM04 (Hirst Magnetic Instruments Ltd, Tesla House, Tregonigie, Falmouth, Cornwall, UK).

ELF-EMFs were calibrated according to a clinically approved Fisiofield Mini magnetotherapy device (Fisioline Co, Verduno, Italy). Technical features were as follows: i) commercial classification: instrument for magnetotherapy; ii) technical classification: medical device class I type BF; iii) medical device class: IIa (93/42/EEC directive); iv) applicable regulations: EN

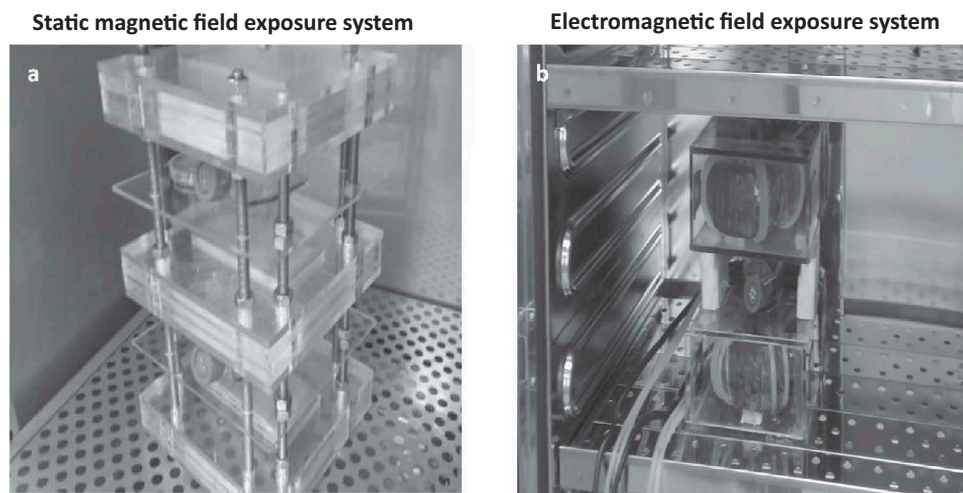


Figure 1. Exposure system: a) a static magnetic field (SMF) that could apply various intensities of a magnetic field according to the distance that was adjusted by a screw and b) an electromagnetic field (EMF) that can apply various intensities (0–100 G) and frequencies (0–100 Hz).

60601-1 (IEC 60601-1), CEI 62-5, EN 60601-1-2 (IEC 60601-1-2); v) size: $220 \times 156 \times 100$ mm; vi) weight: 2 Kg; vii) power: 230 V single phase; viii) line frequency: 50–60 Hz; ix) wave form: square wave; x) emission frequency: 1–100 Hz (ELF); xi) MF's peak intensity: 100 G for each applicator; xii) MF's mean intensity: 50 G for each applicator; xiii) output number: 2; xiv) applicators number: 4; and xv) CE certified: this instrument fully complies with specifications dictated by the European Community under 93/42/EEC and 89/336/EEC directives and further modifications. The device was set to generate continuous square waves with 10, 50 or 100 Hz frequencies and 50 or 100 G amplitudes. As shown in Figure 1, the culture flask was placed between two identical coils so that each one was boxed in a Plexiglas chamber with dimensions of $160 \times 160 \times 50$ mm by maintaining a coaxial distance of 40 mm apart from each other. This distance was obtained by interposing between the two chambers, two side thicknesses of polystyrene. A cooling water system allowed avoiding temperature rise based on the continuous recirculation of cold water around each coil. The intensities of MF, either frequency or amplitude, inside the PVC cell culture flasks were constantly equal to the device-emitting intensities. MFs could pass through all materials used to build the exposure system (i.e., Plexiglas, polystyrene and PVC) without any absorption. Cell cultures were always placed on the same shelves of a cell culture incubator where the ambient 50 Hz MF was 9.5 and 6.2 G with the heater on and off, respectively. The background magnetic amplitude in the laboratory areas, where the cells were processed (next to incubators, worktops and cell culture hood), ranged between 0.8 and 1.4 G (50 Hz). All magnetic amplitudes, including the amplitude values generated by the device, were measured by a Gaussmeter GM08 (Hirst Magnetic Instruments Ltd, Falmouth, UK) equipped with a Hall-effect sensor operating within a sensitivity ranging between 0 and 299.9 G, 1 G resolution and 10 G accuracy (Fig. 1.b)

Cell viability

Cells were seeded into 96-well plates at a density of 12000 cells/well in 200 μ l tissue culture medium in triplicate. After 24-h incubation allowing cells to adhere, the cells were treated for 24 h with EMF and carboplatin combination of both or individual. The count of viable cells was determined indirectly using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Active mitochondria in living cells reduce the MTT. Using 96-well plates (Iwaki) with 1 mg/ml MTT (Product Code: M5655-

1G Sigma-Aldrich Co.), 12,000 cells were incubated in DMEM at 37°C and 5% CO₂ for 2 h. The cells were then washed three times using phosphate buffer saline, pH 7.4 (Product Code: 806552-1L, Sigma-Aldrich Co.), and the reduced MTT formazan crystals were dissolved by 1 ml dimethyl sulfoxide (Product Code: D8418-500 ml, Sigma-Aldrich Co.). The optic density (OD) was then read at 490 nm by an Enzyme-Linked Immunosorbent Assay (ELISA) reader (Pharmacia Biotech, Stockholm, Sweden).

Analysis of apoptosis (Western Blot of cleaved caspase-3)

Apoptosis was investigated by Western Blot of the cleaved caspase-3 protein of whole U-87 cells lysate. Western Blots of caspase-3 protein were analyzed in order to study apoptosis progression. For this purpose, 30 μ g of the total protein from cell lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and then electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA) (Towbin et al., 1979). After blockage for 2 h at RT with 5% (v/v), instant nonfat dry milk/Tris-buffered saline solution, containing 0.1% (v/v) tween 20 (TBS-T), and the PVDF membrane was incubated overnight at 4°C with the primary antibody against caspase-3 (Product Codes: SC-56055 – Santa Cruz Biotechnology Inc., Dallas, TX, USA). Specific antibody binding was detected by incubating the membranes with the horseradish-peroxidase (HRP)-conjugated secondary antibody (Product Code: sc-2005 – Santa Cruz Biotechnology Inc.) at RT for 2 h. Luminol (GE Healthcare Bio-Sciences Corp., Piscataway Township, NJ, USA) was used as a chemiluminescent substrate for HRP. The membranes were washed with TBS-T and scanned by the ChemiDoc™ XRS+ imaging system (Bio-Rad, Hercules, CA, USA). OD-based quantification was performed by Image Lab™ 3.0 (Bio-Rad). B-actin (Product Code: sc-81178 – Santa Cruz Biotechnology Inc., Dallas, TX, USA). It has to be noted that the B-actin was used as control, and the caspase3/B-actin ratio in the control group was compared with the caspase3/B-actin ratio in other groups.

Statistical analysis

One-Way Analysis Of Variance (ANOVA) at the 95% confidence level was performed for all experiments and their data are shown in Figures 2, 5, 6 and 7. A post hoc Bonferroni test was performed for comparing different independent groups with each other, and the Student's t-test at the 95% confidence was performed for all experiments and their data are shown in Figures 3 and

Dose and Time Dependent Cytotoxicity of Carboplatin

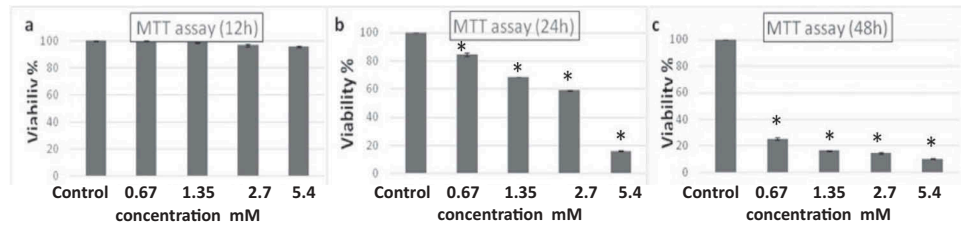


Figure 2. Carboplatin dose and time-dependent dose–response curve of viability (MTT assay); U-87 cells treated with different concentrations of carboplatin at three periods of 12, 24 and 48 h (a, b and c); the star indicates the value significantly different from the control (untreated cells) ($p < 0.05$, $n = 6$).

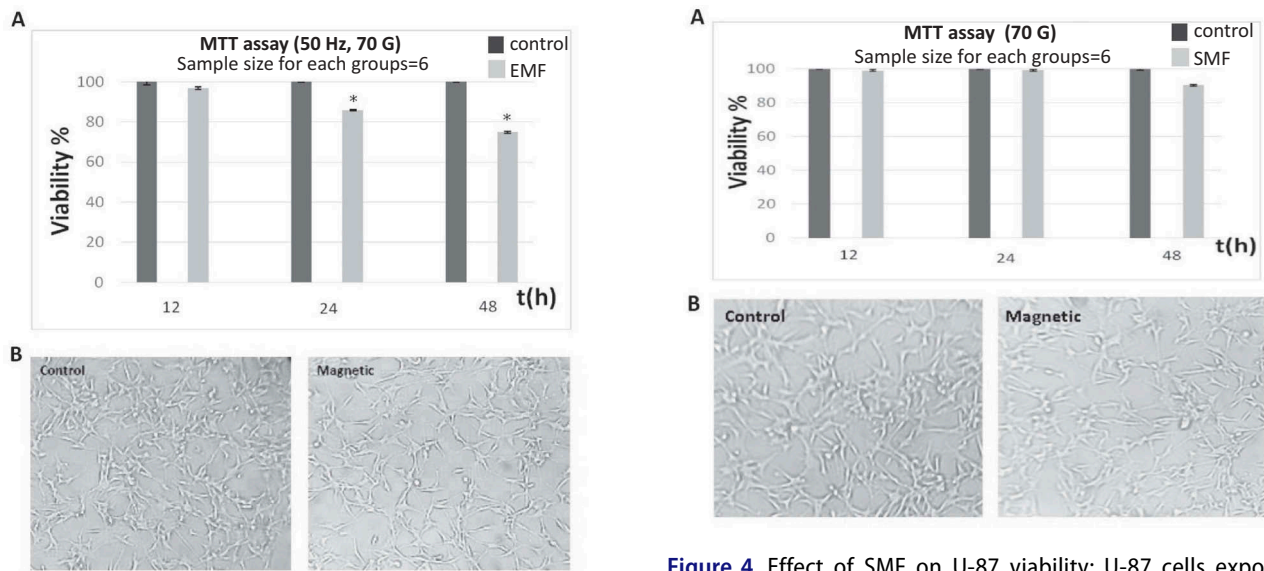


Figure 3. Effect of EMF on U-87 viability; U-87 cells exposed to EMF at intensity 70 G and 50 Hz for three periods of 12, 24 and 48 h: a) values are reported as a percentage of the control-unexposed cells considered as 100%; each value represents the mean $\pm$ SE of five independent experiments, each done four times; the star denotes a value significantly different from the control-unexposed cell at different time points ($p < 0.05$, $n = 6$) and b) light microscope micrographs showing the U-87 cells in the control and electromagnetic exposure group after 48 h.

4. In these cases, two different and independent groups were compared together. p -Values less than 0.05 were considered to be significant.

Results

Evaluation of the effective dose of carboplatin on the cell viability of U-87 cells

In this study, four groups were considered to study the effects of different doses of carboplatin: Group 1: 0.67 mM,

Figure 4. Effect of SMF on U-87 viability; U-87 cells exposed to SMF at intensity 70 G for three periods of 12, 24 and 48 h: a) there was no significant difference at the three periods of 12, 24 and 48 h. The star indicates a value significantly different from the control (unexposed cell) at the different time point ($p < 0.05$, $n = 6$). b) Light microscope micrographs showing the U-87 cells in the control and static magnetic field exposure group after 48 h.

Group 2: 1.35 mM, Group 3: 1 2.7 mM and Group 4: 5.4 mM. Dosimetric evaluation of the drug and its effect on the amount of cellular life was performed using MTT assay in three periods of 12, 24 and 48 h. The results of statistical analysis showed that the doses did not cause IC50% for 12 h. On the other hand, a time interval of 48 h resulted in an increase in the death rate of cells to 90–80%, which was not suitable for selection and further studies. A review of different doses at 24 h showed that a dose of 2.7 mM reduced the cell viability by 50%, and thus this dose was tested for further investigation. The star indicates the value significantly different from the control (untreated cells) ($p < 0.05$, $n = 6$, $F = 6.4$, 6.2 and 6.1 for each time group such as 12, 24 and 48 h, respectively) (Figures 2a, 2b and 2c).

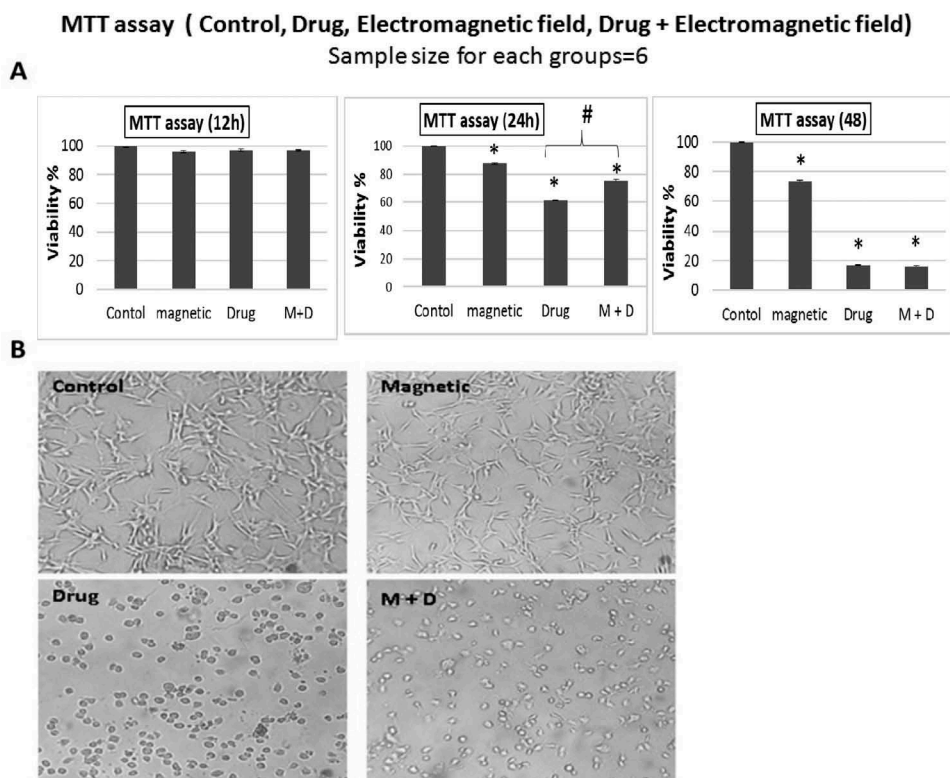


Figure 5. Effect of concomitant carboplatin and EMF on U-87 viability; U-87 cells treated with EMF and carboplatin drug for three periods of 12, 24 and 48 h (a, b and c): A) at 24 h, the magnetic field in combination with the carboplatin drug increased the cell viability about 14%. The star denotes a value significantly different from the control-unexposed cell at the different groups and the square indicates different between drugs and drug+EMF ($p < 0.05$, $n = 6$). b) Light microscope micrographs showing the U-87 cells in different groups: control, EMF, drug and Drug+EMF after 24 h.

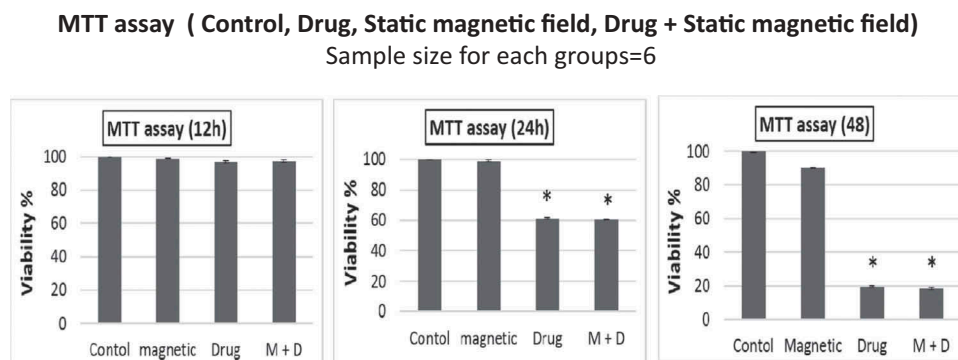


Figure 6. Simultaneous effect of the SMF and carboplatin on viability; U-87 cells treated with SMF and carboplatin drug for three periods of 12, 24 and 48 h (a, b and c): a) at concomitant magnetic field and drug with the drug alone, no significant difference was observed. The star denotes a value significantly different from the control-unexposed cell at the different groups.

Evaluation of the cell viability of U-87 cells under the influence of frequency MF

The effect of the frequency MF at an intensity of 70 G and 50 Hz was investigated on the U-87 cell line in three intervals of 12, 24 and 48 h. The effect of the frequency MF did not differ significantly on the cellular life in the 12 h interval, but in the interval 24 and 48 h showed a significant decrease in cell viability (Figure 3).

Evaluation of the cell viability of U-87 cells under the influence of SMF

In this section, the effect of the 70 G SMF on the U-87 cell line was investigated in three intervals of 12, 24 and 48 h, and the SMF did not show any significant effect after 12, 24 and 48 h in comparison with control (Figure 4).

Caspase-3 Expression
(Control, Drug, Electromagnetic field, Drug + Electromagnetic field) after 24h

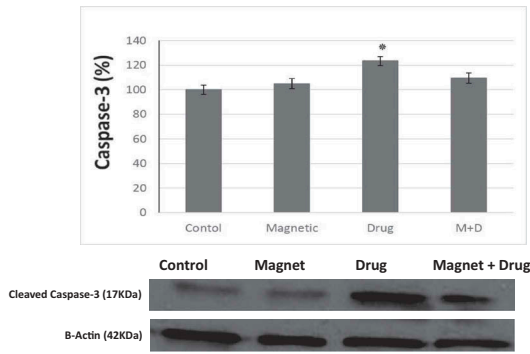


Figure 7. The expression of cleaved caspase-3 protein after 24 h exposure to EMF and carboplatin concomitantly; cleaved caspase-3 protein expression (Western Blots) of U-87 exposed to a 50 Hz, 70 G, EMF and/or treated with 2.7 mM carboplatin for 24 h; values are reported with respect to the optic density (OD) of the control untreated cells, by considering it as 100%. Each value represents the mean $\pm$ SEM. The star indicates the value significantly different from the untreated control cells ($p < 0.05$).

Evaluation of the cell viability of U-87 cells under the simultaneous effect of the extremely low frequency field and carboplatin

The simultaneous effect of the frequency MF and carboplatin drug (2.7 mM) on the U-87 cell line was investigated at three intervals of 12, 24 and 48 h. The results showed no significant difference at interval 12 h, but at 24 h, the frequency MF significantly reduced the effect of the drug, and the combination of the drug and MF increased the cellular viability. At interval 48 h, since the death rate of the cells reaches 90–80%, the MF has no effect on the performance of the drug. The star indicates the value significantly different from the control (untreated cells) ($p < 0.05$, $n = 6$, $F = 4.6$, 4.5 and 4.1 for each time group such as 12, 24 and 48 h, respectively) (Figure 5).

Evaluation of the cell viability of U-87 cells under the simultaneous effect of the SMF and carboplatin

The simultaneous effect of the SMF and carboplatin (2.7 mM) on the U-87 cell line was investigated at three intervals of 12, 24 and 48 h. In none of the time intervals, the MF was effective on the drug and no significant difference was observed. The star indicates the value significantly different from the control (untreated cells) ($p < 0.05$, $n = 6$, $F = 4.4$, 4.2 and 4.7 for each time group such as 12, 24 and 48 h, respectively) (Figure 6).

The simultaneous effect of the extremely low frequency field and carboplatin on the expression of caspase-3 protein expression after 24 h

The frequency MF and carboplatin drug were evaluated based on the expression of caspase-3 protein expression with Western Blot protein. Assay of apoptosis was performed using caspase-3. Caspase-3 protein expression increased significantly in the carboplatin group compared to the control group. Also, in the drug and MF groups, the expression of the protein was increased 23% and 4%, respectively, as compared to the control group. Due to the reduced effect of the drug by frequency MF, the expression of caspase-3 protein in the drug and MF groups decreased simultaneously compared to the drug alone ($p < 0.05$, $F = 4.6$) (Figure 7).

Discussion

The present study was conducted to investigate the effects of frequency and SMFs on the cell viability and apoptosis, as well as the effect of these two fields on decreasing or increasing the effect of carboplatin anticancer drugs. First, the drug was studied in four doses, followed by a medication dose of 50% of the cells' deaths. Dosimetry of the drug was performed at three intervals of 12, 24 and 48 h. Since we did not find the IC50 at interval 12 h and approximately 80–90% of the death occurred in glioblastoma cells at 48 h, these two periods were not investigated. After each time interval, a dose of 2.7 mM for 24 h was evaluated as an effective dose. In a similar study, the effects of carboplatin and tamoxifen were studied. Mastronardi et al. (1998) examined the effect of these two drugs alone and also in combination with the U-138 cell line. In this study, the presence of cells exposed to tamoxifen was shown to inhibit their growth linearly and achieved an IC50% concentration of 12.5 mM. They achieved a carboplatin with an average of 350 mM to IC50%, and the combination of these two drugs increased the cytotoxic effects of both on the cell line (Mastronardi et al., 1998).

The cells were then exposed to a frequency of 50 Hz and 70 G. At this stage, a decrease in cell viability was observed. Also, no significant changes were observed in the survival of the cells by SMFs. Several studies have shown that the MF can activate many types of cells and cause cytokine release (Aldinucci & Pessina, 1998; Cossarizza et al., 1993, 1989; Morandi et al., 1994). The MF can also interact with ions in the membrane cations and proteins; thus, calcium plays a key role in the adjustment of many movements and cellular behaviors (Balcavage et al., 1996).

(Flipo et al., 1998) observed that exposing the lymphocyte cells and spleen macrophages to a SMF with an

intensity of 0.15 T would alter the level of intracellular calcium and cause the cell to travel through the apoptotic pathway. On the other hand, (Raylman, Clavo, & Wahl, 1996) exposed HTB77 and lymphoma CCL86 ovarian cancer cells to a SMF of 7 T for 64 h, which reduced the number of these cells. Also, Buemi et al. (2001) investigated the effects of the SMF by exposing kidney cells and cortical astrocytes to a 0.5 mT field for 2 and 6 d. Finally, a decrease in cell proliferation and an increase in the number of cells with necrotic morphology were observed (Buemi et al., 2001).

In the present work, we also examined the simultaneous effects of SMFs in combination with carboplatin. The carboplatin and SMFs did not show a significant difference in any of the time intervals.

However, in the case of frequency MF and carboplatin drug, a significant difference was observed over a period of 24 h, and the field reduced the effect of the drug in this period. The results of induction of apoptosis also show the expression of caspase-3 protein using the Western Blot technique.

In another study, human colon adenocarcinoma cells were used as a cancer model to investigate the ability of mitomycin C and cisplatin to treat anticancer drugs in the presence of 1.5 MT and 25 Hz MFs. The results showed that the toxicity of these two drugs increased in the presence of the field (Ruiz-Gomez et al., 2002).

Conclusion

In our previous studies, we determined that static and frequency MF decreased and increased the drug efficiency (Akbarnejad et al. 2017a, Akbarnejad et al. 2017b, Vergallo et al., 2014). In this project, we demonstrated that EMF (50 Hz, 70 G) reduced the cytotoxicity of carboplatin on human glioblastoma U-87 cells. Since EMF reduced the apoptotic effect of carboplatin, possibly through a redox regulation mechanism, we have to avoid the coadministration of MF and carboplatin in tumor area, because the MF decreased the toxicity of the drug. However, further studies are needed to find the mechanism of this possible novel therapeutic approach.

Conflict of interest

The authors declare no conflict of interest.

Declaration of interest

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