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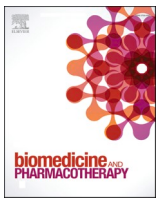

The exposure to extremely low frequency electromagnetic-fields inhibits the growth and potentiates the sensitivity to chemotherapy of bidimensional and tridimensional human osteosarcoma models☆

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ABSTRACT

We previously established a thermodynamical model to calculate the specific frequencies of extremely low frequency-electromagnetic field (ELF-EMF) able to arrest the growth of cancer cells. In the present study, for the first time, we investigated the efficacy of this technology on osteosarcoma, and we applied a precise frequency of the electromagnetic field on three human osteosarcoma cell lines, grown as adherent cells and spheroids. We evaluated the antitumour efficacy of irradiation in terms of response to chemotherapeutic treatments, which is usually poor in this type of cancer. Importantly, the results of this novel combinatorial approach revealed that the specific exposure can potentiate the efficacy of several chemotherapeutic drugs, both on bidimensional and tridimensional cancer models. The effectiveness of cisplatinum, methotrexate, ifosfamide and doxorubicin was greatly increased by the concomitant application of the specific ELF-EMF. Moreover, our experiments confirmed that ELF-EMF inhibited the proliferation and modulated the mitochondrial metabolism of all cancer models tested, whereas mesenchymal cells were not affected. The latter finding is extremely valuable, given the importance of preserving the cell reservoir necessary for tissue regeneration after chemotherapy. Altogether, this novel evidence opens new avenues to the clinical applications of ELF-EMF in oncology.

1. Introduction

Osteosarcoma (OS) is the most common non-hematologic primary bone malignancy originating from mesenchymal tissue among children, adolescents and young adults [1]. The standard therapy for osteosarcoma, comprising surgery and chemotherapy, was established in the 1980s and results in long-term survival in > 60 % of patients presenting with localised disease [2]. Of the patients who present with metastatic disease, 74 % present with lung-only metastases, 9 % with bone-only metastases, and 8 % with both bone and lung metastases [3]. The prognosis for patients with advanced disease is poor, with a 5-year OS of < 30 % [4–6].

The existing treatment regimen of OS comprises neoadjuvant chemotherapy, comprising methotrexate, doxorubicin (adriamycin) and cisplatin (designated as MAP regime) with or without ifosfamide [7] or etoposide [8], followed by surgical excision and adjuvant chemotherapy when the disease is localised. However, several side effects and rare

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Nomenclature

Latin letters

A	Area of the surface of the cell membrane [m^2]
c	Specific heat of cell [J kg^{-1} K^{-1}]
V	Volume of the cell [m^3]

Greek letters

α	Thermal convection coefficient [W m^{-2} K^{-1}]
ν	Frequency [Hz]
ρ	Mass density [kg m^{-3}]

toxicities have been identified for this chemotherapeutic protocol justifying the continuous need for new and more effective anticancer drugs against this type of neoplasia [9–11]. Despite the consensus on the first-line treatment of patients with systemic regimens, when recurrence or progression occurs there is no international agreement on the best therapy for OS patients [7,12]. Indeed, there is no consistent second-line treatment plan for advanced osteosarcoma except for first-line normal doxorubicin and cisplatin-based chemotherapy [4]. Moreover, due to inherent and acquired resistance of osteosarcoma, there are limited drugs after failure to the standard treatment [4]. As only limited therapeutic progress has been made in the last decade [1], novel approaches are needed for patients who demonstrate local or distant relapse and, arguably, for patients who respond poorly to standard chemotherapy.

The beneficial effects of the extremely low frequency electromagnetic field (ELF-EMF) were demonstrated in both in vitro [13] and in vivo [14] studies, in an attempt to exploit electromagnetism for biomedical applications and in cancer therapy [15]. Indeed, the investigation of the biological effects of ELF-EMF has unveiled its influence on several cellular processes, including proliferation, differentiation and apoptosis of tumour cells [16], which can be exploited in cancer treatments. In our recent studies [17,18], we have developed a thermodynamic biochemical model able to select the resonant frequency of ELF-EMF that triggers the maximal entropy variation and causes a metabolic shift, specifically affecting different types of cancer in terms of both cancer growth and motility. Most interestingly, we proved the efficacy of ELF-EMF not only on adherent tumour cells, but also in 3D aggregates [19]. This approach is particularly valuable and promising for future application in vivo, especially in aggressive cancer tissues characterised by lack of therapeutic options, due to their resistance to treatments, as it is the case with osteosarcoma.

In the present study, for the first time we applied a specific frequency of the electromagnetic field on three human osteosarcoma cell lines, grown both as adherent cells and spheroids; we evaluated not only the antitumour efficacy of the exposure to ELF-EMF, but most importantly we tested its effectiveness on the sensitivity to chemotherapeutic treatments, which is an application of the cancer-specific electromagnetic field never investigated before.

2. Materials and methods

2.1. Cell cultures

Three models of human fibroblast isolated from patients with osteosarcoma were bought from American Type Culture Collection (ATCC, Rockville, MD, USA): HOS (CRL-1543), MG-63 (CRL-1427) and SJSA-1 (CRL-2098). As a non-malignant counterpart, the human mesenchymal cells BM-MSC-01 and BM-MSC-02 were obtained from bone marrow, isolated and characterised as previously described [20]. HOS and MG-63 cells were grown in Dulbecco's Mem Nutrient Mix F12 (DMEM F12) (EuroClone S.p.a., Pero (MI), Italy), whereas SJSA-1 cells were grown in RPMI-1640 (Pan Biotech, Bavaria, Germany) culture medium at 37 °C in humified 5 % CO_2 atmosphere supplemented with 10 % Fetal Bovine Serum (FBS) (GIBCO, Thermo Fisher, US) containing 2 mM L-glutamine and 1 % antibiotics (penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

To grow the cells as spheroids, cells were dissociated by trypsin and seeded with a concentration of 5000 cells/well in ultra-low attachment plates (Corning; Lowell, MA, USA) using the same medium explained before. Spheroids were formed after 3 day.

Unless otherwise specified, reagents were purchased from Merck (Milan, Italy), whereas plastic ware was from Falcon (Becton Dickinson, Franklin Lakes, NJ, USA).

2.2. Cell size evaluation, ELF-EMF exposure and chemotherapeutic treatments

On the bases of the resonant thermal effect in biophysics [21,22] the lumped model holds to a characteristic frequency for the ELF-EMF exposure:

$$\nu = \frac{\alpha A}{\rho\ c\ V} \tag{1}$$

where ρ is the density of the cell, c is its specific heat, α is the convective coefficient, V is the cell volume, and A is its surface area. So, a measurement of topological properties (A/V) of the cells is required. The ELF-EMF frequency presents non-thermal effects because we use 0–100 Hz, while thermal effects are generated by high frequencies; consequently, no temperature measures were needed.

Cell sizes were evaluated by photographs taken in different areas of the dish. For each adherent cell type and for cell-derived spheroids, cellular and nuclear sizes were measured using an ImageJ software analysis on 30 images (Sun Microsystems Inc., Palo Alto, CA, USA) and the values were used to calculate the specific frequencies by the thermodynamic model [18,23,24]. During experiments, cells were continuously exposed to the ELF-EMF, which was placed inside an incubator, for the required time and at the specific frequencies (31 Hz for osteosarcoma adherent cells and 11 Hz for spheroids, 2 Hz for mesenchymal cells), whereas control cells were grown in the same incubator under standard conditions. Briefly, the cell culture dishes were placed in the central part of the apparatus, made of two independent couples of coaxial coils made of 200 loops. The experimental setup was placed in the incubator inside a box that shielded the apparatus from the background magnetic field. The outer coils were supplied with a direct current (DC) that provided a constant magnetic field of 45 μT, the average value of the earth's magnetic field [17]. The inner coils were connected to an AC current generator that produced a square wave signal at the specific frequencies calculated by the mathematical model and at the intensity of 100 μT. The experimental setup had been previously described [17,19].

For chemotherapeutic experiments, cells were cultured for one day with ELF-EMF exposure and another day of exposure in presence or absence of either cisplatin (Sandoz, Italy), methotrexate (Teva, Italy), ifosfamide (Holoxan) (Baxter, Italy) or doxorubicin (Merck, Milan, Italy).

2.3. Proliferation assay

The effect of ELF-EMF on the cell growth of the different human cancer cell lines was determined in a 96-multiwell plate by bromodeoxyuridine/5-bromo-2′-deoxyuridine (BrdU) incorporation assay (Roche Applied Science, Penzberg, German) following the manufacturer's instructions, after three days of incubation with or without exposure to ELF-EMF. The data collected were averaged for each experimental condition, and each experiment was repeated three times. In some experiments, the cells exposed to ELF-EMF were also treated with 1 mM N-acetyl cysteine (NAC). Real-time polymerase chain reaction (qRT-PCR)

Cells were seeded on 6-multiwell plates and they were cultured for 2 days in standard conditions or exposed to ELF-EMF. The cells were washed with PBS and total RNA was extracted with TRIzol® (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). One μg of total RNA were reversely transcribed into cDNA, in a final volume of 20 μl, using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The RT-PCR primers were designed with NCBI/ Primer-BLAST, synthesized by Sigma (Milan, Italy). Quantitative PCR was carried out in a final volume of 20 μl using the SensiFAST™ SYBR® Hi-ROX Kit (Bioline Srl, Trento, Italy) with specific primers for the quantitation of the following human genes: cytochrome c subunit 2 (COXII, fwd 5'-TCTGGTCAGCCCAACTCTCT-3′, rev 5'-CCTGTGATCCACCAGAAGGT-3′), cytochrome c oxidase subunit 4 (COXIV, fwd 5'-CGAGCAATTTCCACCTCTGT-3′, rev 59-GGTCAGCCGATCCATATAA-39), ATP synthase subunit beta (ATP5B, fwd 5'-GTGGGCTATCAGCCTACCCT-3′, rev 5'-CAAGTCATCAGCAGGCACAT -3′), mitochondrial ATP synthase F0 subunit 6 (MT-ATP6, fwd 5'-CCAATAGCCCTGGCCGTAC-3′, rev 5'-CGCTTCCAATTAGGTGCATGA-3′), uncoupling protein 1 (UCP 1, fwd 5′-CTGGAATAGCGGCGTGCTT-3′, rev 5′-AATAACACTGGACGTCGGGC-3′), uncoupling protein 2 (UCP 2, fwd 5′-GGTGGTCGGAGATACCAAA-3′, rev 5′-CTCGGGCAATGGTCTTGTAG-3′), and beta 2-microglobulin (β2M, fwd 5'-AGCAAGGACTGGTCTTTCTATCTC-3′, rev 5'-ATGTCTCGATCCCACTTAACTA-3′) [19,25,26]. PCR amplification was 1 cycle of denaturation at 95 °C for 2 min, 40 cycles of amplification including denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 30 s. The quantification of each sample was carried out comparing each PCR gene product with β2M, used as reference gene to normalize the cDNA in different samples. Data were analysed using the 2_ΔΔCT method. Analysed transcripts exhibited high linearity amplification plots ($r > 0.98$) and similar PCR efficiency, confirming that the expression of each gene could be directly compared. The specificity of PCRs was confirmed by melt curve analysis. Nonspecific amplifications were never detected.

2.4. Cell viability assays

Cell viability was assessed using the (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) MTT assay [27]. 2500 cells/well were seeded into 96-well plates in 100 μL culture medium, and they were cultured one day in standard conditions or in the presence of ELF-EMF exposure and then 24 h with different pharmacological drugs in the absence or in the presence of ELF-EMF exposure. Then, MTT (0.5 mg/mL) was added to each well, and the supernatants were removed after 4 h of incubation at 37°C. The absorbance values at 570 and 630 nm were determined using a microplate reader Victor 3 from PerkinElmer Life Sciences (Waltham, MA, USA) after the solubilization of the formazan crystals using 100 μL dimethyl sulfoxide (DMSO).

In addition, the cells grown on 96-multiwell plates in the same experimental conditions were stained for 1 h at 37 °C in culture medium containing Neutral Red solution, washed three times with phosphate-buffered saline solution (PBS) and rinsed with stop buffer (4.02 g trisodium citrate in 153 mL water (H_2O), 0.8 mL hydrochloric acid (HCl) 0.1 N in 86 mL H_2O and 25 mL of 95 % v/v methanol). The absorbance was read at 540 nm, and the cell viability was evaluated by measuring the percentage of cells stained with neutral red dye, as previously reported [28].

The chemotherapeutic drugs IC50 values, evaluating the concentration of a drug necessary to inhibit 50 % of cellular functions, were calculated by GraphPad Software as cell viability on osteosarcoma adherent cells.

2.5. Measurement of total cellular ROS production

HOS, MG-63 and SJSA cells were seeded on 6-multiwell plates and cultured for two days in standard conditions or exposed to ELF-EMF. Unstimulated cell was also treated for three hours with 100 μM menadione, which is a known generator of ROS, as a positive control of the assay. The cells were washed, detached in 1 mL PBS by scraping, resuspended in 1 mL of PBS and loaded for 15 min with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is a cell-permeable probe that is cleaved by nonspecific intracellular esterases to form DCFH, which is further oxidized by ROS to form the fluorescent compound dichlorofluorescein (DCF). After the incubation, cells were washed twice with PBS to remove the excess probe, and total DCF fluorescence was determined at an excitation wavelength of 504 nm and an emission wavelength of 529 nm, using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT) [29]. The fluorescence value was normalised to the protein content and expressed as relative to the control. Experiments were performed in triplicates and repeated three times.

2.6. Measurement of mitochondrial membrane potential (ΔΨm)

Membrane potential was determined by evaluating fluorescence shifting of JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), a mitochondrial dye staining mitochondria in living cells in a membrane potential-dependent fashion, as previously reported [25]. Briefly, cells were incubated with JC-1 (2 μg/mL final concentration) at 37 °C for 30 min. The amount of JC-1 retained by 10,000 cells per sample was measured at 530 nm (FL-1 green fluorescence) and 590 nm (FL-2 red fluorescence) with a flow cytometer and analysed with Cell Quest Alias software. The ratio FL2/FL1 was evaluated to determine ΔΨm. Experiments were performed in triplicates and repeated three times.

2.7. Statistical analysis

Statistical analysis of data was performed using ANOVA test with Tukey's post-hoc correction or by an unpaired two-tailed Student's t-test, as required. P values < 0.05 were considered significant and indicated. All data were expressed as the mean ± SEM of three independent experiments.

3. Results

3.1. ELF-EMF exposure inhibits cell growth of human osteosarcoma adherent cells and cell-derived spheroids

BrdU assay of control and treated cells was performed to evaluate the inhibition of cellular proliferation after 3 days of exposure to specific frequencies of the ELF-EMF. HOS, MG-63 and SJSA-1 osteosarcoma adherent cells were sensitive to the calculated specific frequency (31 Hz) (Fig. 1A).

The effect of the electromagnetic field was also assessed on two primary batches of mesenchymal cells deriving from two different BM donors, BM-MSC-01 and BM-MSC-02, the non-tumour counterpart of osteosarcoma. The mathematical model calculated the specific frequency for these cells which was 2 Hz. Unfortunately, the attempt of exposing the cells to the 2 Hz frequency revealed a technical limit of the device, since at this frequency there was an overheating which compromised cellular functionality and, thus, masked the effect attributable to the electromagnetic field alone. However, we demonstrated that 31 Hz was not the effective frequency to block mesenchymal cells cell proliferation, on the opposite this frequency was able to inhibit cancer growth and was even stimulatory on one mesenchymal cell preparation (Fig. 1B). In conclusion, our experiments demonstrated the specificity of the calculated frequency in targeting only cancer cells.

Notably, a different specific frequency of 11 Hz was efficient in significantly decreasing the growth of the HOS, MG-63 and SJSA-1 cell-derived spheroids (Fig. 1C). In fact, as the cell morphology in spheroids was unique and quite different from adherent cells, our mathematical model calculated the most powerful frequency of ELF-EMF for these

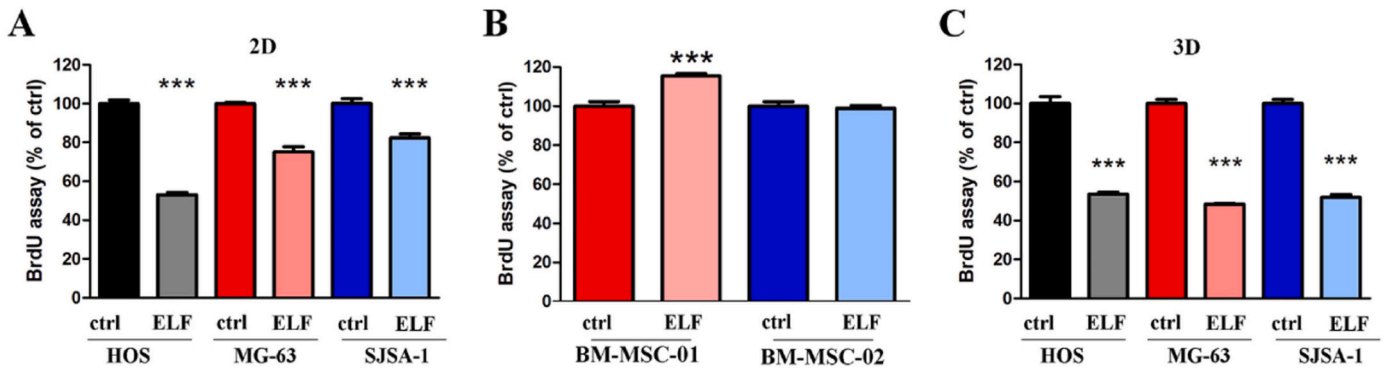

Fig. 1. The exposure to the specific electromagnetic wave inhibits the proliferation of human cancer. After three days of growth in standard condition (ctrl) or in presence of ELF-EMF at the specific frequencies (ELF), the proliferation of HOS, MG-63 and SJSA-1 cancer cells grown as 2D (panel A) and 3D model (panel C), and their non-malignant counterpart BM-MSC-01 and BM-MSC-02 cells (panel B), was analysed by BrdU incorporation. The values of the treated cells are expressed as the percentage of their respective controls (ctrl). The data represent the means ± SEM of three independent experiments. ***$p < 0.001$ compared to the control.

cells. These results demonstrated the specificity of the applied ELF-EMF and confirmed the effect of the exposure also on the three-dimensional osteosarcoma models, which mimic real tumour conditions.

3.2. *Mitochondrial respiration is increased by ELF-EMF exposure in human osteosarcoma adherent cells and cell-derived spheroids*

We assessed the mRNA levels of several proteins involved in mitochondrial respiratory activity and energy production, such as mitochondrial respiratory complexes, ATP synthase and uncoupling proteins, in both adherent and 3D models of HOS, MG-63 and SJSA-1 cells. The 48 h exposure to ELF-EMF both at 31 Hz for adherent cells, and to 11 Hz for spheroids, enhanced the transcription of the subunits necessary for the mitochondrial activity (Fig. 2 and Fig. 3). Indeed, ELF-EMF exposure of 2D cell cultures at the specific frequency of 31 Hz significantly increased the mRNA levels of cytochrome c oxidase subunit 2 (COXII) and subunit 4 (COXIV), which are two subunits of the respiratory complex IV, whose transcripts are, respectively, of mitochondrial and nuclear origin (Figs. 2A and 2B). In addition, two subunits of ATP synthase were upregulated in their nuclear (ATP5B) and mitochondrial

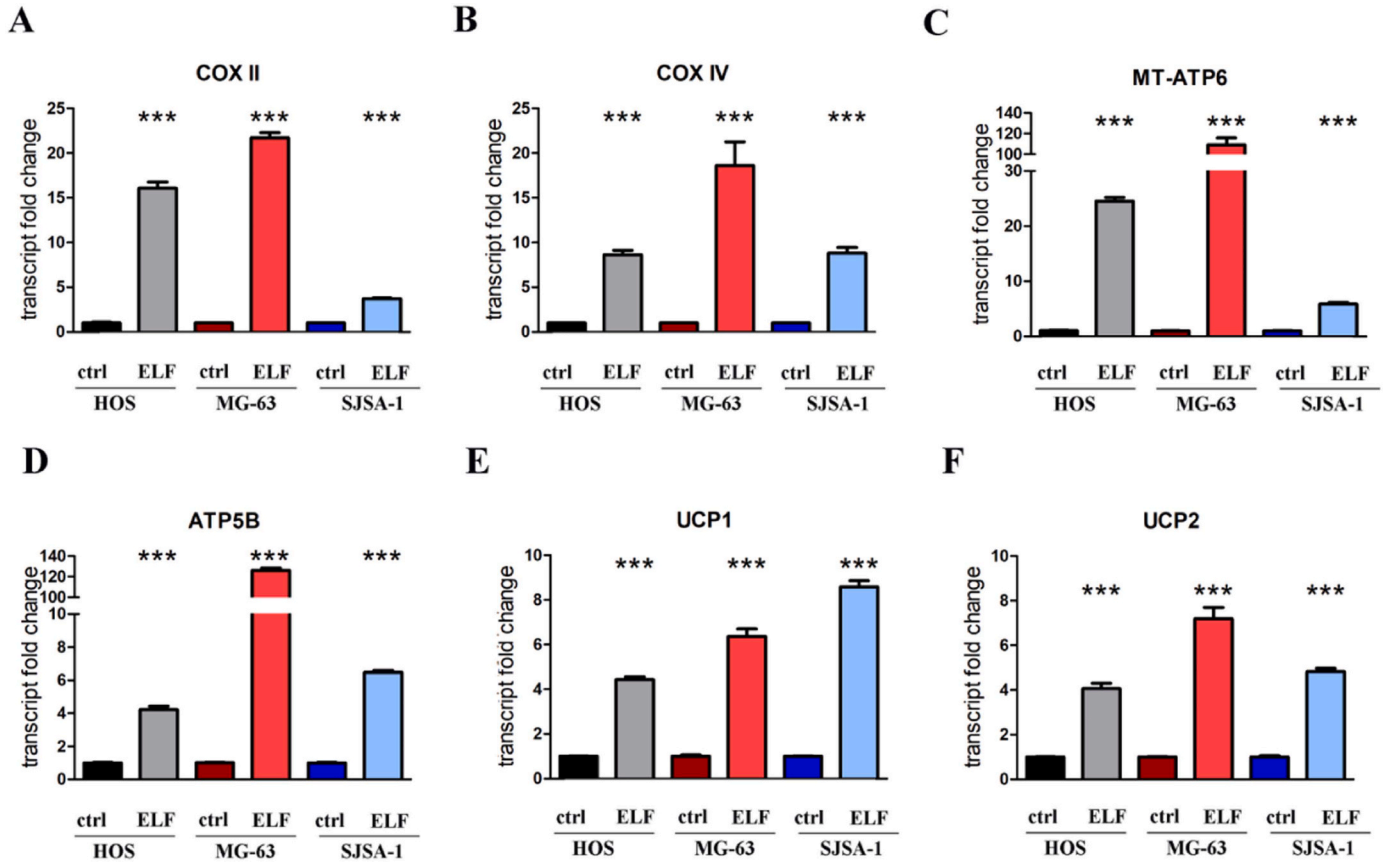

Fig. 2. Exposure to ELF-EMF of osteosarcoma cell lines grown as 2D model increases mitochondrial activity. RT-PCR analysis of two subunits of the respiratory chain COX II and COX IV, two subunits of ATP synthase MT-ATP6 and ATP5B, and the uncoupling proteins UCP1 and UCP2. The expression of transcripts was evaluated in untreated cells (ctrl) and cells treated at the specific frequency (ELF). Fold changes versus control are plotted on the graph. The data represent the means ± SEM of three independent experiments. ***$p < 0.001$ compared to the control.

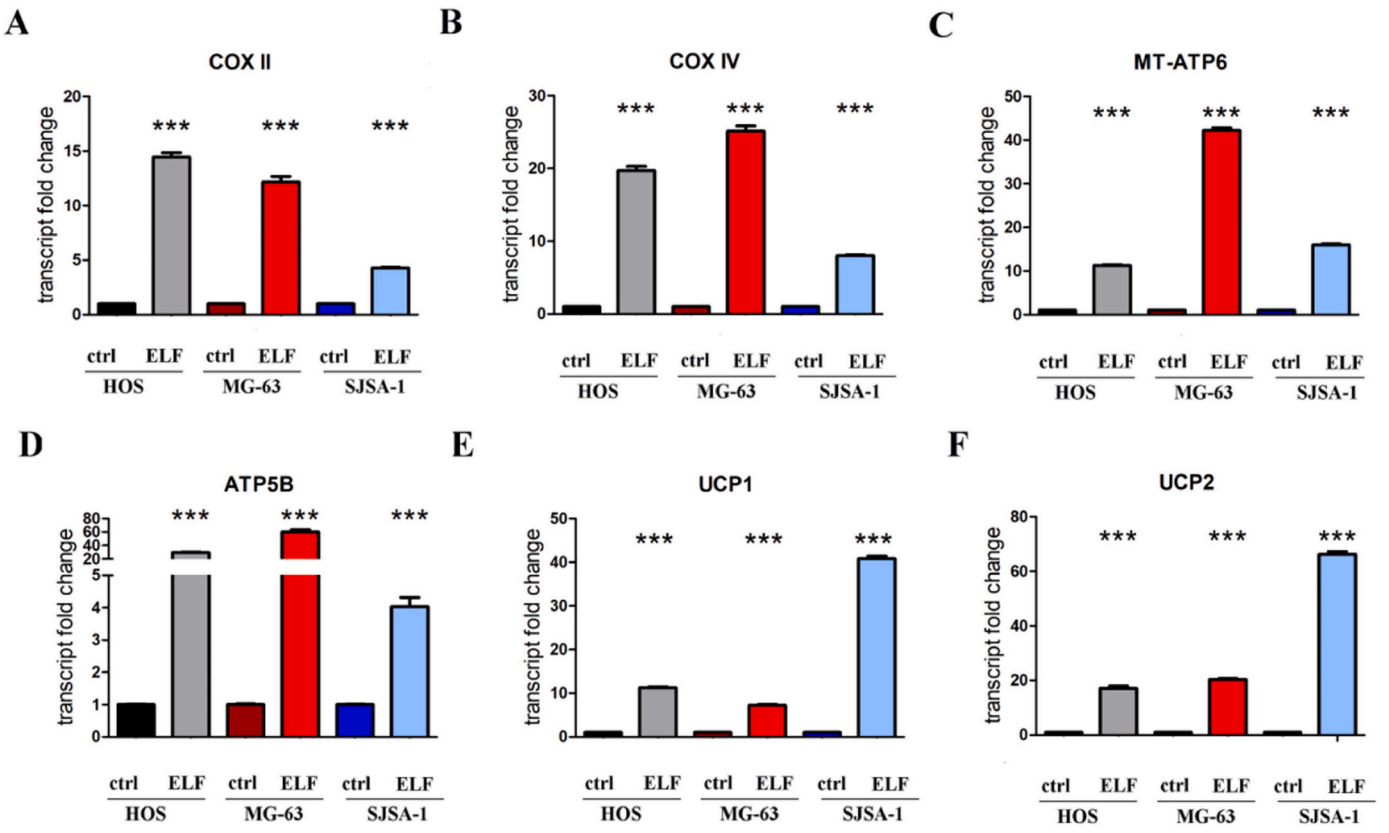

Fig. 3. Exposure to ELF-EMF of osteosarcoma cell lines grown as 3D model increases mitochondrial activity. RT-PCR analysis of two subunits of the respiratory chain COX II and COX IV, two subunits of ATP synthase MT-ATP6 and ATP5B and the uncoupling proteins UCP1 and UCP2. The expression of transcripts was evaluated in untreated cells (ctrl) and cells treated at the specific frequency (ELF). Fold changes versus control are plotted on the graph. $^{***}p < 0.001$ compared to the control.

transcription (MT-ATP6) (Figs. 2C and 2D). This suggests that selective ELF-EMF frequency strongly stimulates the respiratory elements. Similarly to respiratory elements, also the uncoupling proteins UCP1 and UCP2 were upregulated in their transcription by ELF-EMF (Figs. 2E and 2F). Because the effect of ELF-EMF is exerted both on respiratory elements and on uncoupling proteins, any increase of proton gradient is readily dissipated and mitochondrial membrane potential does not change, as verified by JC-1 assay and shown in Supplementary Fig. S1. Interestingly, the frequency of 31 Hz did not modulate the metabolism of normal cells; in fact, the transcription of the mitochondrial respiratory elements of mesenchymal preparations was not affected by the cancer-specific ELF-EMF, as shown in Supplementary Fig. S2.

Moreover, considering the mitochondrial metabolism of cell-derived spheroids after ELF-EMF exposure, the same effect was obtained when HOS, MG-63 and SJSA cells aggregated in a spheroid mass. Indeed, in spheroids, the levels of COX2, COX4, ATP5B, MT-ATP6, also, of the uncoupling proteins UCP1 and UCP2 transcripts were upregulated after 3 days of exposure to the ELF-EMF at 11 Hz (Fig. 3). These results confirm that the electromagnetic field at selective frequency stimulates the metabolism of both two-dimensional and three-dimensional tumour models, and most importantly our experiments demonstrate the specificity of the calculated frequency in targeting only cancer cells.

3.3. *Intracellular ROS production is not affected by ELF-EMF*

Because we detected the ELF-EMF-triggered induction of mitochondrial respiration, we measured the production of radical oxygen species (ROS), which is a bystanding effect of cellular respiration, to investigate a plausible new mediator of the biological effect exerted by the electromagnetic field. In our analysis, we discovered that unexposed SJSA-1 cells displayed a ROS content lower than the other cell lines. Moreover, when HOS, MG-63 and SJSA-1 cell lines were exposed to the frequency of 31 Hz for 1 day, a modest but significant increase in ROS content was detected in our experimental conditions (Fig. 4A). However, the small additional quantities of ROS that were formed during ELF-EMF exposure were not enough to suggest a possible toxic and antiproliferative effect exerted by ROS. Indeed, despite the modest ROS increase, cell viability was not modified by the exposure to the electromagnetic field, as demonstrated by MTT assay (Fig. 4B). To confirm that the slight increase of ROS levels was not mediating the inhibitory effects of ELF-EMF on growth, we also performed an experiment in the presence of 1 mM N-acetyl cysteine (NAC), an antioxidant molecule, and the BrdU proliferation assay did not reveal any effect of NAC on the proliferation of cells exposed to the electromagnetic field (Fig. 4C). Altogether, these data suggest that ROS are not the mediators of the ELF-EMF anti-tumoural effect and that the antiproliferative and stimulatory effects of ELF-EMF are not due to cytotoxicity.

3.4. *ELF-EMF exposure improves the efficacy of chemotherapeutic agents both in human osteosarcoma adherent cells and cell-derived spheroids*

To evaluate the effect of ELF-EMF on the sensitivity to chemotherapeutics, all the human adherent osteosarcoma cell lines and cell-derived spheroids were exposed to the frequency of 31 and 11 Hz, respectively, and treated with standard chemotherapeutic drugs. After one day of exposure to the electromagnetic field followed by another day of exposure in the presence of chemotherapeutic treatments, the efficacy of the in vitro chemotherapeutic and ELF-EMF treatment was evaluated by measuring cell viability. The MTT assay was employed to test the sensitivity to cisplatin, methotrexate and ifosfamide, whereas the neutral red assay was carried out to evaluate the response to doxorubicin, in standard growth conditions in the absence or the presence of

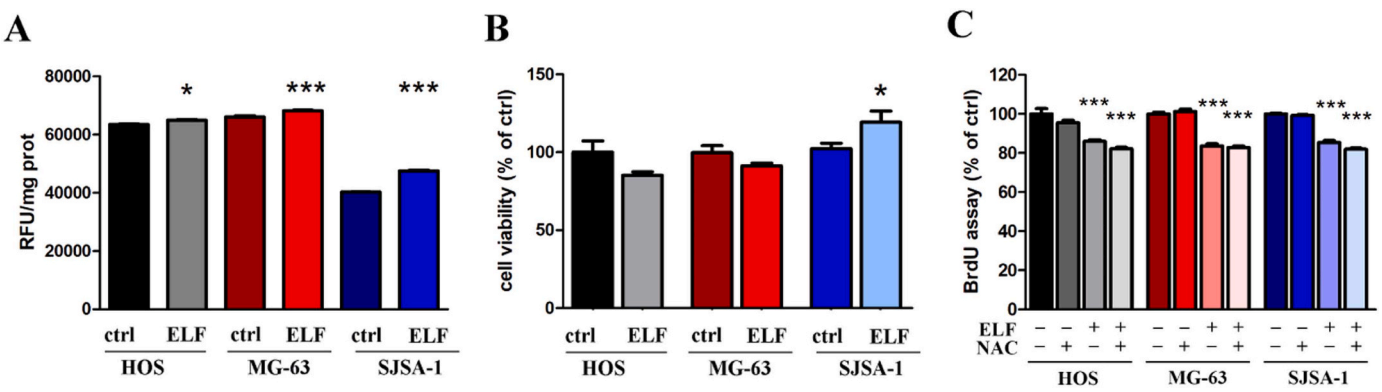

Fig. 4. ROS do not mediate the anti-tumoural effects of ELF-EMF. (A) After 24 h of exposure of the adherent cell lines to the specific electromagnetic wave (ELF), ROS production was evaluated by DCF fluorescence relative to protein content. (B) Cell viability was measured by MTT assay. (C) The proliferation of the three cancer cell lines was tested by BrdU incorporation in presence of 1 mM of the antioxidant molecule N-acetyl cysteine (NAC). The data represent the means ± SEM of three independent experiments. *$p < 0.05$ compared to the control; ***$p < 0.001$ compared to the control (panels A and B) or to the unexposed and untreated cells (panel C).

ELF-EMF. The experiments on human adherent osteosarcoma cell lines revealed that exposure to ELF-EMF lowered the IC50 of each tested anticancer agent (as shown in Table 1).

On osteosarcoma spheroids, the tested doses of drugs were those calculated as IC50 values in adherent cells and the concentration a hundred times lower than IC50 values. The experiments of cell viability revealed that also in spheroids the exposure to ELF-EMF in presence of each tested anticancer agents significantly decreased cell viability if compared with a single treatment (Fig. 5).

When observed by light microscopy, the spheroids that were exposed to ELF-EMF alone and in combination with 1 μM cisplatin appeared not only smaller, but also deteriorated and surrounded by cell loss and cellular debris (Supplementary Fig. S3). In the standard growth conditions, the three-dimensional mass was very compact (ctrl) and the outline was clearly visible, unlike the treated spheroids, in which the compactness and boundaries were less clear. Spheroid degeneration was most evident with the combined treatments, particularly in SJSA-1 cell line, partially resistant to cisplatin.

4. Discussion

In this study, we tested the effect of the very low-frequency electromagnetic field on a particularly malignant tumour model, the osteosarcoma. Due to the frequent failure of standard treatments, innovative treatments of osteosarcoma are eagerly awaited. In the past we have tested various tumour models, demonstrating the ability of our thermodynamic model to calculate the cell-specific frequency for each cancer, and with the biochemical approach, we analysed the biological impact of the electromagnetic field [17,19,24].

In this study, for the first time, we have revealed another important application of ELF-EMF in oncology, namely the ability to enhance the effect of chemotherapy. First, we confirmed that the ELF-EMF frequency specifically calculated for this tumour had the expected antiproliferative and metabolic effects, as reported in our previous works [17,18,23,24] for other cancer types but newly investigated in this study on osteosarcoma; then, we discovered that in the osteosarcoma model, the electromagnetic field significantly reduced the half-maximal inhibitory concentration of four drugs widely used in clinical practice. This means that the same antitumour efficacy can be achieved using a lower dosage of the chemotherapeutic agent if the drug is administered in association with the exposure to ELF-EMF. Notably, administering a lower dosage of cytotoxic drugs would reduce the negative impact of chemotherapy. Moreover, it was exciting to verify that this effect could also be obtained in 3D cultures, which grow with a structure closer to the architecture of the tumour mass. An additional important breakthrough of this research was the verification that the tumour-specific frequency does not affect healthy cells; the exposure to ELF-EMF did not arrest the growth of mesenchymal cells, and this is another advantage of this approach in the treatment of bone cancer since stem cells are an important reservoir of cells necessary for the tissue's reparative response and even mesenchymal stem cell transplantation is proposed as post-surgery therapy of bone cancer [30]. Bone regeneration after cancer therapy relies on the survival and activities of resident stem cells [31], therefore the combined treatment of lower doses of chemotherapy together with an exposition to specific frequencies of ELF-EMF sparing mesenchymal cells would greatly improve the recovery from the disease.

The study is strengthened by experimental verification on three osteosarcoma cell lines with different characteristics, grown both in 2D and 3D. In particular, the effect obtained on SJSA-1 cells is interesting since these cells are described in the literature as resistant to chemotherapeutics [32]. In our experimental setting, these cells required a concentration of cisplatin and methotrexate higher than the other cell lines to achieve half-maximal cytotoxicity, and their sensitivity to the drugs was greatly enhanced by the exposure to the electromagnetic field, both in 2D and 3D cultures. In this study, the enhancement of the cytotoxic effect of chemotherapeutics was also confirmed by microscopic observation of 3D models, in which the additive effect of ELF-EMF and chemotherapy was particularly evident for SJSA-1 cells.

The mechanism of action of ELF-EMF does not appear to be mediated by the modest but significant increase in ROS production, which is a consequence of the positive modulation of mitochondrial respiration

Table 1
IC50 values calculated for each anticancer agent tested for 24 h on adherent cell lines exposed or not exposed to ELF-EMF.

Chemotherapeutic agent	Adherent Cell Line	IC50 (μM) in absence of ELF-EMF	IC50 (μM) in presence of ELF-EMF	Decrease factor
Cisplatin (CP)	HOS	0.1232	0.0248	4.952
	MG63	0.6855	0.0893	7.669
	SJSA-1	0.9032	0.1845	4.895
Methotrexate (MTX)	HOS	1.163	0.0468	24.840
	MG63	0.4216	0.0554	7.602
	SJSA-1	1.590	0.1484	10.714
Ifosfamide (IFO)	HOS	0.4820	0.3544	1.360
	MG63	0.8123	0.5628	1.443
	SJSA-1	0.4486	0.1379	3.253
Doxorubicin (DOXO)	HOS	0.5240	0.1146	4.572
	MG63	0.3580	0.1799	1.999
	SJSA-1	0.2834	0.1022	2.773

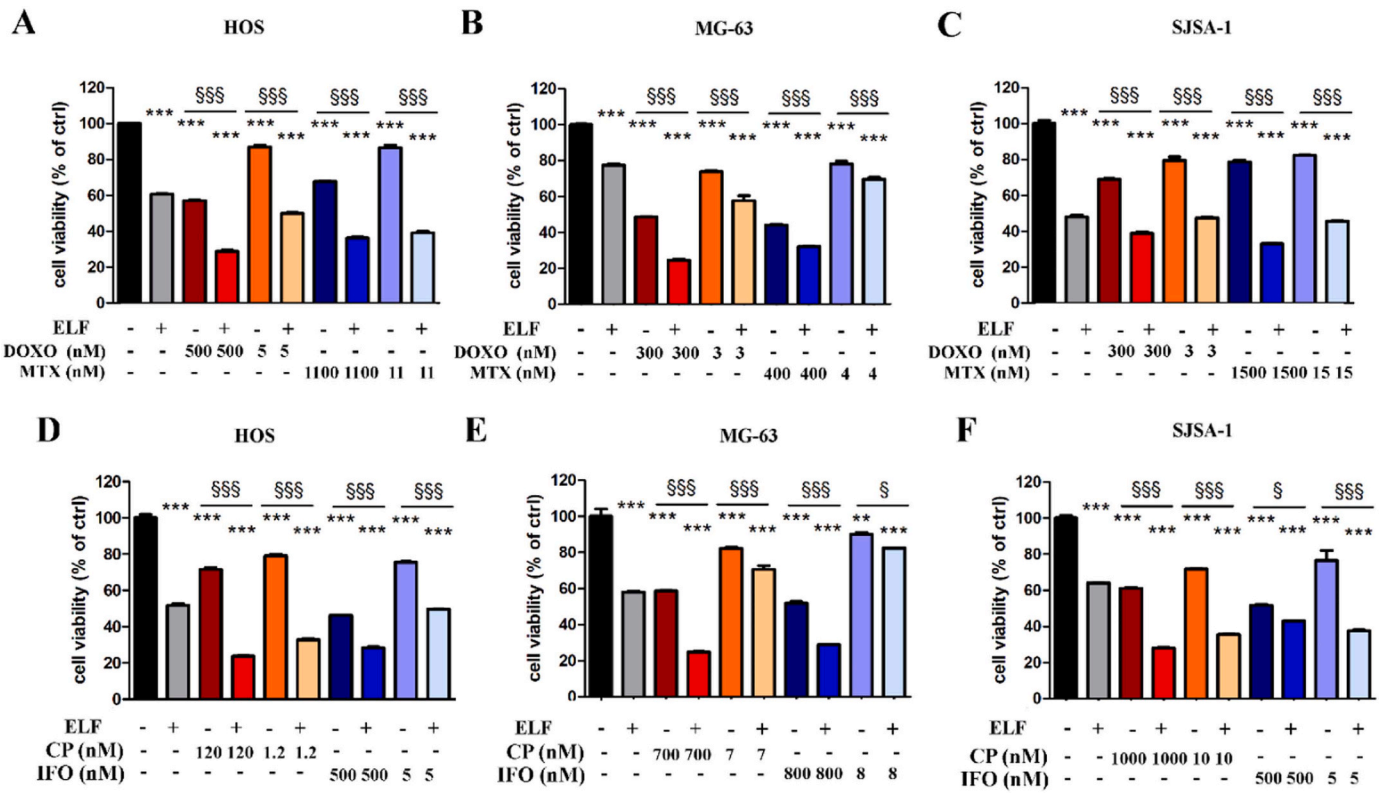

Fig. 5. Exposure to ELF-EMF potentiates the cytotoxicity of antitumoural drugs. 3D models of osteosarcoma cell lines were exposed for 24 h to ELF-EMF only (ELF) or treated for additional 24 h with the indicated doses of drugs: doxorubicin (DOXO), methotrexate (MTX), cisplatin (CP), or ifosfamide (IFO). Cell viability was measured by MTT assay and data are expressed as percentage of the unexposed and untreated cells. The data represent the means ± SEM of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the unexposed and untreated cells; §$p < 0.05$ and §§§$p < 0.001$.

triggered by ELF-EMF. In previous work, we demonstrated that the variation in calcium fluxes is associated with greater energy production at the mitochondrial level [16], which is essential for the cell to maintain the homeostasis of ionic fluxes, perturbed by the electromagnetic field. On this basis, the data of the present work suggest that the increased production and consumption of energy triggered by ELF-EMF hamper the efficient protection from chemotherapy since it is known that the pharmacological efflux at the basis of both the natural and acquired resistance to chemotherapy is an ATP-dependent phenomenon and it is well known to be one of the mechanisms used by tumour cells to deal with cytotoxic effects of chemotherapy agents [33,34]. Alternatively, it can be suggested that the mitochondria could be the target of both the metabolic stress exerted by ELF-EMF and the pharmacological toxicity of the drugs; the convergence could damage the organelle and lead to cellular death. Both hypotheses need to be further investigated, but this novel study opens interesting new perspectives on the use of ELF-EMF in oncology, in association with standard but very often poorly effective therapies, especially in the advanced disease phase. It should be noted that the effect on the spheroids is greater than that observed on 2D cultures, probably due to an enhanced metabolic stress to which the cells within the 3D mass would be subjected (notoriously hypoxic, [35]). Furthermore, the effectiveness of spheroids suggests the ability of the electromagnetic field also to affect drug-resistant stem cells, which are abundant in these models, with progenitor properties that are responsible of tumour relapses and metastasis [36].

In conclusion, the results of this study reveal an interesting new application of the extremely low-frequency electromagnetic field in oncology. The possibility of enhancing the effect of the chemotherapy is particularly valuable in chemo-resistant cancers, but also in chemo-sensitive tumours the application of the field could allow a much lower pharmacological dosage and thus limit the toxic effects of the treatments.

CRediT authorship contribution statement

Debora Fino: Project administration, Funding acquisition. **Katia Mareschi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Data curation. **Loredana Bergandi:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **Giulia Grisolia:** Writing – review & editing, Writing – original draft, Software, Project administration, Methodology, Investigation, Formal analysis. **Antonio Ponzetto:** Validation, Supervision. **Umberto Lucia:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Francesca Silvagno:** Writing – review & editing, Writing – original draft, Validation, Supervision, Data curation, Conceptualization. **Sebastian Dorin Asaftei:** Validation, Supervision. **Franca Fagioli:** Writing – review & editing, Writing – original draft, Validation, Supervision. **Alessia Giovanna Santa Banche Niclot:** Writing – review & editing, Writing – original draft, Visualization, Data curation. **Elisa Tirtei:** Writing – review & editing, Writing – original draft, Visualization, Data curation. **Elena Marini:** Writing – review & editing, Writing – original draft, Visualization, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117162.

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