

Effects of Acute and Chronic Low Frequency Electromagnetic Field Exposure on PC12 Cells during Neuronal Differentiation

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Key Words

ELF-EMFs neuronal differentiation • PC12 cells • Reactive oxygen species • Intracellular Ca^{2+} variations

Abstract

Background/Aims. The purpose of this study was to provide information about the *in vitro* neurogenesis during cell exposure to extremely low frequency electromagnetic fields (ELF-EMFs) of different intensities and durations using pheochromocytoma-derived cell line (PC12 cells) as neuronal model. **Methods.** Proliferative rates and neurogenesis were tested by colorimetric assay and morphological analysis, respectively; reactive oxygen species (ROS) levels and intracellular Ca^{2+} variations monitored using single cell videomicroscopy. **Results.** The long-lasting ELF-EMF exposure (0.1-1.0 mT) did not appear to significantly affect the biological response (proliferation and neurogenesis). However, during the acute ELF-EMF exposure (30 min), in undifferentiated PC12 cells, there were increased ROS levels and decreased catalase activity, that, conversely, resulted increased after chronic exposure (7 days) at 1.0 mT. Acute exposure (0.1-1.0 mT) affected the spontaneous intracellular Ca^{2+} variations in undifferentiated cells, in which

basal intracellular Ca^{2+} resulted increased after chronic exposure. In addition acute exposure affected cell response to a depolarizing agent, while basal membrane potential was not changed. **Conclusion.** Even if further studies remain necessary to identify the ROS/intracellular Ca^{2+} cross-talking pathway activated by ELF-EMF exposure, we support the hypothesis that ROS and Ca^{2+} could be the cellular “primum movens” of the ELF-EMF induced effects on biological systems.

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Introduction

The functional link between the presence of electromagnetic fields (EMFs) and modifications to physiological and/or pathological processes in living organisms, including man, has been known for a long time and has even been used in therapy [1]. However, the processes induced by EMFs on living matter are far from being fully explained.

The biological effects of extremely low frequency electromagnetic fields (ELF-EMFs), from domestic and industrial networks, have been demonstrated by an in-

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creasing number of experimental findings confirming that under certain conditions, non-ionising electromagnetic energy can influence physiological processes in organisms [2-10]. Various cellular processes and systems can be affected by ELF-EMF exposure: e.g. cell membranes, general and/or specific gene expression, cell proliferation and differentiation, metabolic pathways, and many other physiological mechanisms that are correlated directly or indirectly with cell viability [11-17].

In man, the nervous system represents one of the immediate systems interacting with the environment. Alterations or the incorrect functioning of its cells can lead to the induction of neoplastic mechanisms, particularly for the more sensitive stages, such as during development (when neuronal networks are forming) or decline due to age.

Neuronal cells are vulnerable to the presence of ELF-EMFs and there are experimental evidences that ELF-EMFs interfere with their functional activity [18, 19].

In previous studies, it has been found that exposure to ELF-EMFs increases proliferation and inhibits programmed cell death in human neuroblastoma and rat pituitary cells [20], on the other hand, in rat chromaffin cells and in neural stem cells ELF-EMFs promoted neurogenesis and neuronal differentiation [21, 22].

Blackman et al. demonstrated that, in PC12 cells, ELF-EMFs stimulated neurite outgrowth in a flux-density-dependent manner between 2.2 and 4.0 μT , reaching an apparent stimulation plateau between 4.0 and 40.0 μT ; while no effects were seen at 0.9 μT or lower intensities [23]. Also McFarlane and colleagues reported, in PC12 cells, a neurotogenic effect induced by ELF-EMFs after 23h exposure but in a different intensity range (4.35–8.25 μT); while exposure to higher fields (8.25–15.8 μT) had no significant effect on neurite outgrowth [24].

In summary, the effects of exposure to ELF-EMFs on cells can be difficult to interpret because findings are often contradictory. Difficulties may arise because effects are often small and derive from different electromagnetic field parameters (e.g. intensities, duration of exposure and their combinations). Confounding variables can also arise from differences in the cell type.

In most cellular models the ELF-EMF-induced effects are found in the intracellular calcium variations [25-28].

In addition, a remarkable amount of data support another interesting theory that finds in chemical reactions involving free radical production, the target of ELF-EMFs [29]. In fact in the last years, some data from literature

have described redox-related cellular changes, following exposure to ELF-EMFs [10, 27, 30-32].

The purpose of this study is to contribute to improve information about cell responsiveness and *in vitro* neurogenesis during exposure to ELF-EMFs of different intensities and durations using pheochromocytoma-derived cell line (PC12 cells) as neuronal model.

In particular, this study focused on: i) the determination of modifications of biological parameters (morphology, proliferation and differentiation) induced by exposure to ELF-EMFs; and ii) the possible involvement of intracellular calcium-dependent oxidative stress machinery in cell response to ELF-EMF exposure.

Materials and Methods

Equipment and cell exposure parameters

The electromagnetic fields were generated by two different devices: a solenoid for chronic exposure and a pair of Helmholtz coils for acute one. This choice was due to the technical and physical characteristics of the devices in order to generate a homogeneous field to which the dishes, containing cells, were exposed. In addition the device for chronic exposure should be located within an incubator without altering the environmental conditions [33]. The parameters of the generated fields (including field homogeneity in the zone of cell exposure) were tested with an apparatus specifically designed, built and set [33].

The ELF-EMFs were generated with a frequency of operation of 50 Hz and magnetic field density (B) between 1 μT and 1 mT \pm 2%, defined along the centre line, using specifically designed and built Helmholtz coils (Oersted Technology Corp., Troutdale, OR, USA). The minimum step of B was 1 μT ; the generated magnetic field was centred with a volume of homogeneity that was spherical with a radius of 10 mm corresponding to the centre of the microscope stage where the dishes were located for the experiments; the polarization of the field was horizontal; and the coils were perpendicular to the ground. The pair of Helmholtz coils ($r=445$ mm, distance between coils 400 mm), located in the working zone of a confocal microscope and connected to an Elgar Electronics power supply (CW-1251P; Elgar Electronics, San Diego, CA, USA), produced homogeneous magnetic fields over the specified volume under test corresponding to the cell chamber. The coil carrier was nonmagnetic with maximal thermal and mechanical stability. The disturbance in the generated electromagnetic field by the electrical connection between the two coils and/or by the power supply connection was 2% of the minimum level of the magnetic field within the volume of homogeneity. Thermal stabilization of the device was achieved by free convection (ambient $T=25^\circ\text{C}$).

The Helmholtz coils were fitted to a confocal imaging system consisting of an inverted microscope Zeiss Axiovert 100 (Zeiss, Jena, Germany) with an oil immersion 63 \times /1.25 Plan

Neofluar objective and a Bio-Rad Model MCR-1024 ES hardware system (Bio-Rad Microscience Ltd, Hemel Hempstead, UK) equipped with an argon/ krypton mixed-gas laser. Images were acquired with Laser Sharp 3.1 software (Bio-Rad Microscience).

A horizontal solenoid (Oersted Technology Corp.), designed and built to deliver variable, homogeneous, sine-wave alternating current magnetic fields with 50-Hz frequency and intensities ranging from 0.1 to 1.0 mT \pm 2%, was used to expose a large number of cells simultaneously. The horizontal cylindrical solenoid (length 340 mm, diameter 113 mm) was mounted on a nonmagnetic supporting base and powered with an Elgar Electronics power supply (CW-801P). The solenoid was used in an incubator (5% CO₂ and 37°C) for long time continuous cell exposures (1-7 days) during which the solenoid's eventual added temperature was negligible. Cells were cultured in plastic dishes transparent to the ELF magnetic field.

As the presence of ELF-EMFs in nature and in the daily life is extremely variable for intensity and duration, the planned experiments considered two modalities of temporal exposure: immediate (acute, up to 30 min) and long lasting (chronic, up to 7 days), with a variable scale of intensity of the field (0.1 mT to 1.0 mT, 50 Hz). The ELF-EMF intensity range of 0.1-1.0 mT was selected as it is the most common exposure in urban environments and in domestic appliances, and 0.1 mT is the upper reference level currently recommended by the European Council for general safe public exposure (1999/519/CE).

Reagents

Unless otherwise indicated, cell culture chemicals were obtained from Gibco BRL (Invitrogen, S. Giuliano Milanese, Italy), cell culture ware was from Becton-Dickinson Falcon (Sacco Srl, Cadorago, Italy), and reagents and standards were from Sigma-Aldrich (Milan, Italy).

Cell culture and morphology

PC12 cells, (batch F-13518; ATCC, MD, USA), established from a rat pheochromocytoma [34], were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum (HS), 5% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1 mM L-glutamine (Growth Medium, GM). Prior to stimulation, if not otherwise indicated, cells were synchronized by overnight serum starvation. The neuronal differentiated phenotype was obtained by culturing cells in GM supplemented with 1,5% HS (DM: differentiation medium) and 50 ng/ml NGF (Alomone, Israel) for 7 days. Both undifferentiated and differentiated PC12 cell phenotypes were routinely maintained at 37°C in a 5% CO₂ humidified atmosphere.

Undifferentiated and differentiated PC12 cells were exposed to 50 Hz ELF-EMFs (0.1-1.0 mT) and analysed during the exposure (acute effects) or after long exposure times (days). Control cells were sham-exposed cultures under the switched off coils or solenoid.

The morphological analysis of the control and exposed cells was performed by optical microscopy. Cell cultures, at selected times, were washed with phosphate-buffered saline (PBS) and then fixed for 10 min in 3.7% paraformaldehyde (Sigma) at room temperature. Photomicrographs were taken by

a Nikon ECLIPSE TS100 inverted microscope equipped with a Nikon Coolpix 4500 digital camera (Nikon Instruments Inc., Melville, NY, USA).

Cell growth and viability

The analyses of cell viability and growth were performed by Trypan Blue exclusion test and colorimetric assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, USA) respectively.

Briefly, 10⁴ PC12 cells were plated in 96-well plates in a final volume of 200 µl in GM per well. At selected times in absence or presence of ELF-EMFs, MTT was added to each well to a final concentration of 0.5 mg/mL. The plates were incubated for 3 h at 37 °C and then centrifuged at 500 g. The supernatants were removed and discarded and 200 µL dimethyl sulfoxide (DMSO, Sigma-Aldrich) were added. After incubating for 30 min at 37°C, the absorbance was determined by spectrophotometry (SpectraMAX 190) at a wavelength of 560 nm [35].

The trypan blue exclusion assay was performed by staining cells with trypan blue (0.5% in PBS) and counting in a Burkert camera. Blue stained cells were considered non-viable.

Neurite outgrowth assay

Neurite outgrowth was determined using the method previously described by Gysbers et al [36]. In brief, PC12 cells were plated on poly-L-lysine-coated wells in 24-well plates at a concentration of 10⁵ cells per well. After overnight serum starvation, The cells were incubated in presence of 50 ng/mol NGF (Sigma-Aldrich) in absence or presence of ELF-EMFs. At selected times, the medium was aspirated from the plates, taking care not to disturb the cells. The cells were immediately fixed in 3.7% paraformaldehyde for 10 min. Fixed cultures were examined under an inverted Zeiss Axiovert 100 microscope (Jena, Germany) equipped with a phase contrast lens. For each treatment, the total number of cells and the number of cells with neurites were counted on three randomly selected sample fields from each of five individual wells that had received identical treatments. A neurite was defined as a process extending from the cell, which was at least one cell body [37, 38]. The mean values (\pm S.E.M.) were then determined for each treatment.

Videoimaging on single cell

ROS levels, Ca²⁺ signalling, membrane potential variations were evaluated in both undifferentiated and differentiated PC12 cells with H₂DCF-DA (dichlorodihydrofluorescein diacetate, Molecular Probes, Eugene, OR, USA), Fluo-4 AM (Molecular Probes) or DIBAC₄ (*bis*-(1,3 dibutylbarbituric acid) trimethine oxonol, Molecular Probes) respectively. PC12 cells (3.0 \times 10⁴ cells/cm²) were seeded in GM onto round, poly-L-lysine-coated sterile coverslips (diameter 25 mm) 24 h before ELF-EMF exposure. Differentiated PC12 cells, prepared by incubating for up to 7 days in DM, as described above, were similarly treated. Immediately before exposure to ELF-EMFs, cells on coverslips were incubated 30 min at 37 °C in normal external solution (NES, 140 mM NaCl, 2.8 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.3) containing 10 µM H₂DCF-DA or 5

μM Fluo-4 AM or 500 nM DIBAC₄. After incubating with probes, the cells were rinsed with NES and observed for up to 30 min during exposure to ELF-EMFs (0, 0.1, and 1.0 mT) generated by Helmholtz coils as described under Equipment. During the entire experimental procedure, all confocal system parameters (Kr/Ar laser intensity, photomultiplier, and pinhole size) were kept constant. The loaded cells were excited at a wavelength of 488 nm; emitted fluorescence was collected at 522 nm using a bandpass filter (bandwidth \pm 32 nm). Green fluorescence was recorded every 2 or 30 s for 30 min.

The fluorescence values recorded during the first 3 min of observation were taken as the mean basal fluorescence level. In the case of cultures exposed to ELF-EMFs, Helmholtz coils were turned on after the first 3 min of observation.

At the end of each experiment, for level ROS, hydrogen peroxide (H_2O_2) was used as internal control and to test cell susceptibility to an oxidizing agent, for intracellular Ca^{2+} , ionomycin and EGTA were added sequentially to obtain maximum and minimum values, respectively, for changes in membrane potential, KCl was used as internal control.

Images were sampled, stored offline, and analyzed. Traces per total cell area were calculated as f/f_0 , where f is the fluorescence emission of a single loaded cell at times ranging from 0.03 or 0.5 to 30–40 min, and f_0 is the mean fluorescence intensity value of the same cell calculated from all images acquired during the first 3 min or before turning on the coils.

For each experimental condition, at least 10 coverslips were analyzed.

Spectrofluorimetric determination of basal $[\text{Ca}^{2+}]_i$

After 7-day ELF-EMF exposure, the cells were detached and washed by gentle centrifugation, then resuspended in NES and loaded with 5 μM Fura 2-AM for 30 min at 37°C and treated for 5 min with 250 μM sulphhydrylase then collected in the quartz cuvette and analysed using LS-5B Perkin-Elmer spectrofluorimeter. The $[\text{Ca}^{2+}]_i$ was calculated using the formula: $[\text{Ca}^{2+}]_i = K_d (F - F_{\text{min}}) / (F_{\text{max}} - F)$, where F_{max} and F_{min} were the fluorescences recorded at the end of each experiment, after the addition of 1 μM Ionomycin and 5 mM EGTA respectively; K_d (224 nM) is the dissociation constant of Fura 2 for calcium [39].

Western blotting

The PC12 cells were grown in absence or presence of ELF-EMFs (0.1 mT or 1.0 mT) for various times (1–7 days). At the end of this treatments, the cells were rapidly washed with cold PBS, and lysed in a solution containing: 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 50 mM NaF, 40 mM β -glycerophosphate, 5 mM EDTA, 1% Triton X-100, 200 μM sodium orthovanadate, 100 $\mu\text{g}/\text{mL}$ PMSF, 10 $\mu\text{g}/\text{mL}$ leupeptin, 5 $\mu\text{g}/\text{mL}$ pepstatin A, and 10 $\mu\text{g}/\text{mL}$ benzamidine. After five min of vortexing, the samples were centrifuged at 10000 \times g for 10 min at 4 °C. The protein content of the resulting supernatant was quantified using a colorimetric assay (Bio-Rad Laboratories Srl, Milan, Italy). After resuspension in Laemmli buffer (25 mM Tris-HCl, pH 6.5, 8% SDS, 10% glycerol, 5% β -mercaptoethanol and 0.003% bromophenol blue), the samples (40 μg protein) were denatured by 5–10 min of boiling and then applied to 10%

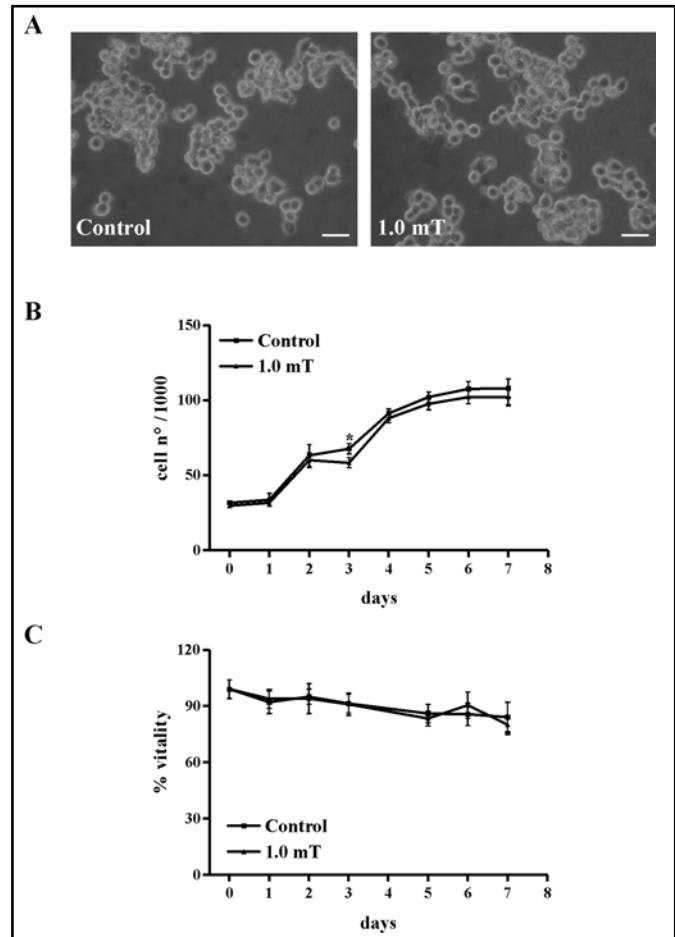


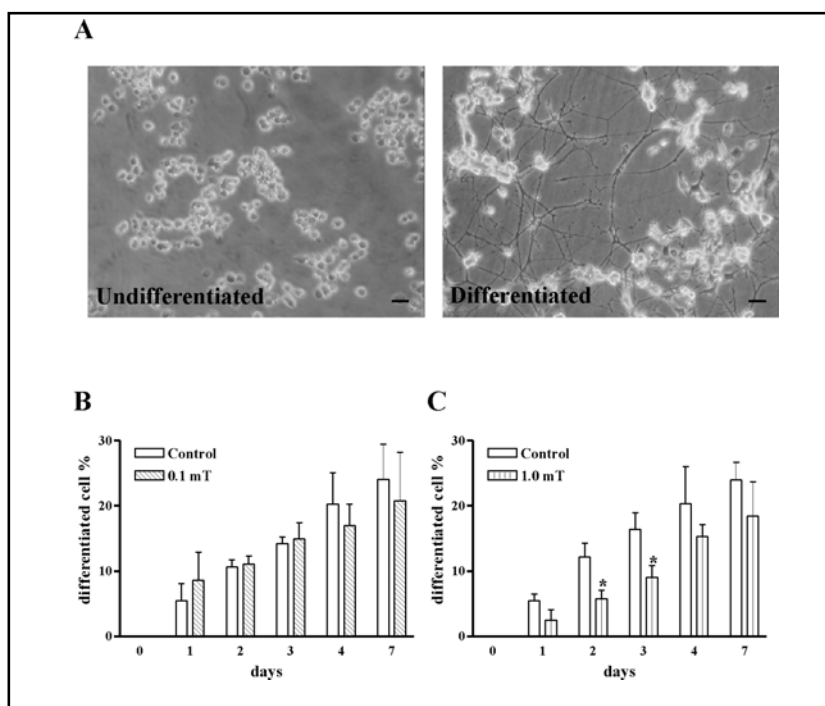
Fig. 1. PC12 cell morphology and proliferation. Panel A shows representative images of undifferentiated PC12 grown for 7 days in the absence (Control) or presence of 1.0 mT ELF-EMF, as indicated. Scale bar=20 μm . Panel B and C report cell proliferation and viability respectively. Growth curves of PC12 cells grown in absence (Control) or presence of 1.0 mT ELF-EMFs, as indicated. The data are means \pm S.D., $n = 8$, * $p < 0.05$. The percentage of cell vitality is expressed as the percentage of living cells with respect to the total number of PC12 cells grown in absence (Control) or presence of 1.0 mT ELF-EMFs, as indicated. The data are means \pm S.D., $n = 8$.

polyacrilamide gels (SDS-PAGE). The proteins were then transferred to polyvinylidene difluoride membranes (Hybond-P Amersham-Pharmacia Biotech). The protein levels of GAP43, MAP2 and actin were revealed with specific antibodies: mouse anti-GAP43 monoclonal antibody; mouse anti-MAP2 monoclonal antibody (Sigma-Aldrich); mouse anti-actin monoclonal antibody (ICN Biomedicals, Eschwege, Germany). Anti-actin antibody was used as a loading control. These antibodies were then revealed through chemiluminescence (ECL, Amersham-Pharmacia Biotech).

Enzyme activities

Catalase activity was determined in samples derived from sonicated undifferentiated, differentiating and differentiated PC12 cells, suspended in 20 mM Na-phosphate buffer (pH 7.0)

Fig. 2. PC12 cell neuronal differentiation. In panel A, representative PC12 cell images of undifferentiated phenotype (left panel) and after 7 days of differentiation in the presence of 50 ng/mL NGF (right panel) are reported. Scale bar=20 μ m. Panel B shows the percentage of PC12 cells with neurites in non-exposed control cultures and in those exposed to ELF-EMFs at 0.1 mT or 1.0 mT. The data are means \pm S.E.M., n=10; * p<0.05.



containing 1 μ g/ml pepstatin, 1 μ g/ml leupeptin, and 100 μ M phenylmethylsulfonyl fluoride (PMSF) as protease inhibitors. Total protein concentrations were quantified using a colorimetric assay (Bio-Rad Laboratories). Catalase activity was determined based on the decrease in absorbance due to H₂O₂ consumption ($\epsilon = -0.04 \text{ mM}^{-1} \text{ cm}^{-1}$) measured at 240 nm, according to the method described by Morabito and colleagues [27]. The final reaction volume of 1 ml contained 100 mM Na-phosphate buffer (pH 7.0), 12 mM H₂O₂, and 100 μ g of sample.

Statistical analyses

Statistical analyses of the data were carried out with Prism version 4 Graphpad software (GraphPad Software, San Diego, CA, USA), using analyses of variance via the t-test for the unpaired data.

Results

Biological effects induced by ELF-EMFs

To verify the possible effects of ELF-EMFs on cell morphology, growth and differentiation, we cultured PC12 cells into the turned off (sham control) or powered sole-noid.

The morphology was monitored using a transmission light microscope, collecting images from randomly selected fields in samples grown in the absence and presence of ELF-EMFs for 7 days. During this period, and for ELF-EMFs of up to 1.0 mT, there were seen no appreciable morphological changes (Fig. 1A).

During the exposure period the proliferative ability and the presence of dead cells in the cell population were

also monitored through a colorimetric method and using a vital dye (see materials and methods). The exposed PC12 cells showed growth curves similar to that of control cells with the exception of a slight transient decrease in the cell number, seen after three day exposure to 1.0 mT ELF-EMFs (Fig. 1B). The number of non-viable cells did not change in all tested conditions, as shown by the representative data reported in Figure 1C. The 0.1 mT exposure did not affect nor morphology, proliferation rate or the number of dead cells (data not shown).

The determination of the effects of the ELF-EMFs on neuronal differentiation was carried out by analysing the growth of neurite-like processes. This was monitored in PC12 cells incubated the presence of NGF, whose differentiation mechanisms and timing in this model were well described [34]. The quantitative analysis, reported in Figure 2B-C, revealed that the number of cells bearing neuritis, increased with time in the presence of NGF. The cells exposed to a 0.1 mT field showed a time course similar to that of the control cells (Fig. 2B). Conversely the cells exposed to 1.0 mT, showed a slowing rate of the differentiation process, with a significantly lower number of cells bearing neuritis, than the controls, during the first days of differentiation. This effect was transient as no significant difference was seen with respect to the control within 4-7 days (Fig. 2C). During NGF-induced differentiation and in absence or presence of ELF-EMFs (0.1 or 1.0 mT) the protein expression levels of GAP43 and MAP2, two specific neuronal markers were assayed. As reported in Figure 3, GAP43 and MAP2

Fig. 3. GAP43 and MAP2 expression levels. Representative Western blots showing the levels of neuronal markers in differentiating PC12 cells incubated in absence (C) or in presence of 0.1 or 1.0 mT ELF-EMFs for 3 and 7 days (d), as indicated. The graphs collect densitometric analyses from three independent experiments and expressed as means \pm S.E.M.

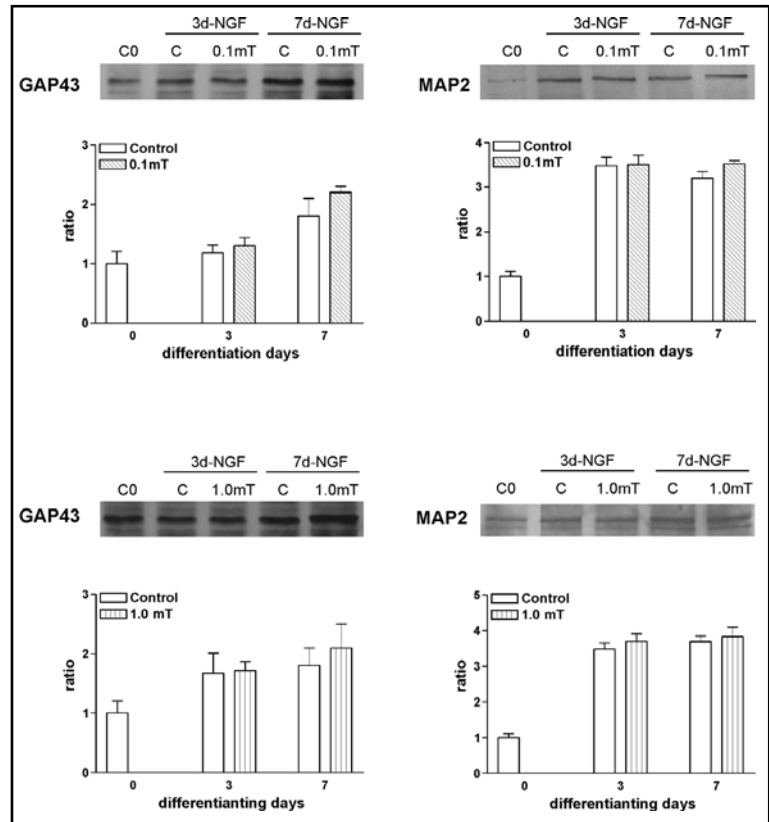
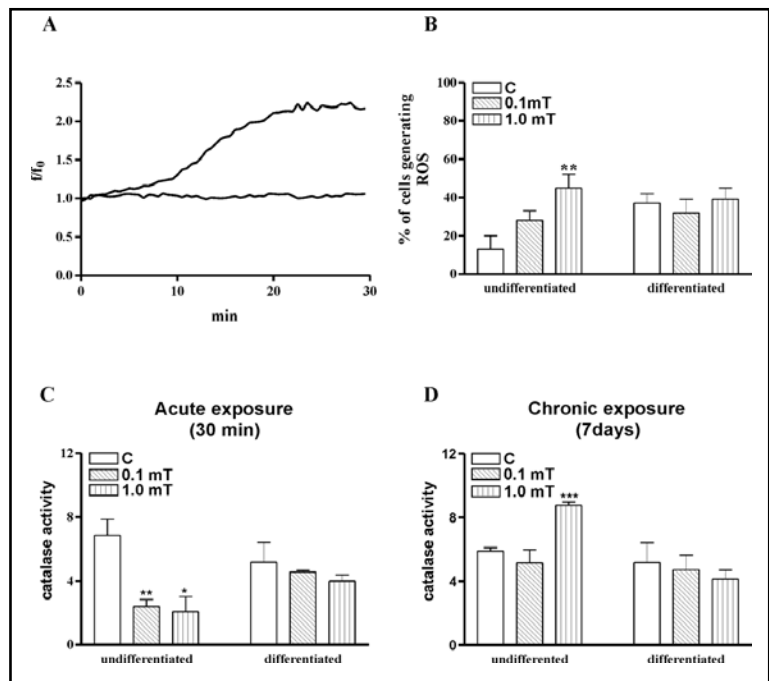


Fig. 4. Oxidative markers in PC12 cells. In panel A, the traces represent temporal analyses of the fluorescence (f/f_0) of single PC12 cells. Each trace is representative of a single cell behaviour. For each experimental condition (non-exposed or exposed cells to 0.1 or 1.0 mT ELF-EMFs), at least 10 fields (each containing 60-80 cells) in different coverslips were analysed. In panel B, quantitative analyses of DCF-loaded undifferentiated and differentiated PC12 cells are expressed as percentage of cells producing ROS during the experimental observation in different experimental conditions (with turned off coils (control), in presence of 0.1 or 1.0 mT ELF-EMFs, as indicated). The bar are means \pm S.E.M., $n=10$. ** $p<0.005$. In panels C and D, the specific activity of catalase, expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein and measured on cell homogenates from undifferentiated and differentiated PC12 cells after different experimental conditions, as indicated, are shown. The bar are means \pm S.E.M., $n=5$. * $p<0.05$; ** $p<0.005$.



levels did not change in exposed cells in respect to control ones.

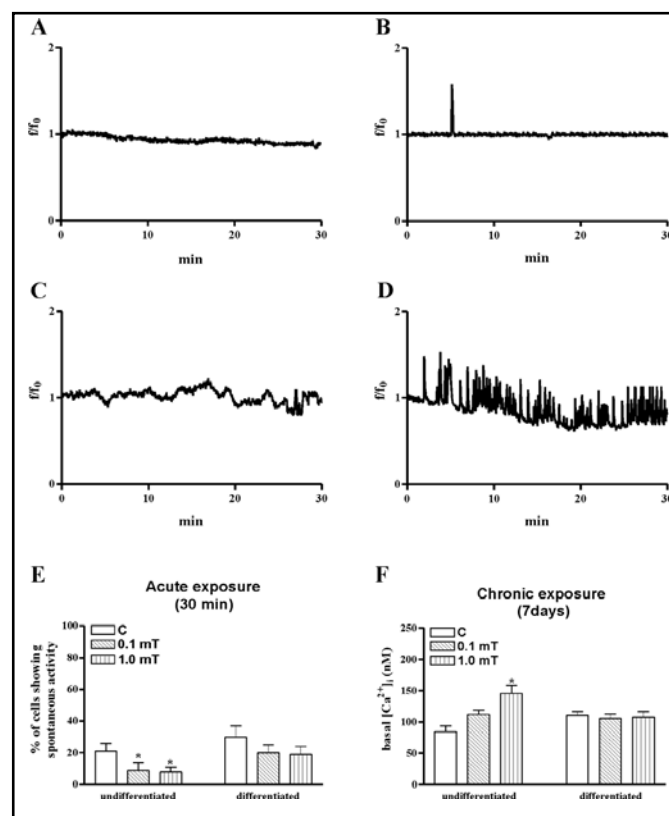
Intracellular responses during ELF-EMF exposure- ROS levels and catalyse activity

We investigated the possible induction of a “stressogenic” event caused by the ELF-EMFs during

the exposure time in undifferentiated or neuron-like PC12 cells. ROS generation was monitored during continuous cell exposure to ELF-EMFs (0.1 or 1.0 mT) using single-cell confocal microscope imaging, employing $\text{H}_2\text{DCF-DA}$ as a specific ROS probe (see Materials and Methods).

In PC12 cell populations it was possible to observe or a stable intracellular ROS levels or spontaneous in-

Fig. 5. Intracellular calcium. Panel A depicts no cellular activity; panel B, a single isolated intracellular Ca^{2+} spike; panel C, instable intracellular calcium levels; panel D, high frequency intracellular Ca^{2+} waves. In particular, undifferentiated PC12 cells showed in equal amount the A, B and C kinetics, while NGF-differentiated PC12 revealed intracellular Ca^{2+} variation depicted in A, B and D. The graphs are representative of a single cell behaviour. For each experimental condition (non-exposed or exposed cells to 0.1 or 1.0 mT ELF-EMFs), at least 10 fields (each containing 66-81 cells) in different coverslips were analysed. Panel E reports quantitative analyses of Fluo4-loaded undifferentiated and differentiated PC12 cells, expressed as percentage of cells showing spontaneous activity during the experimental observation in different experimental conditions (with turned off coils (C), in presence of 0.1 or 1.0 mT ELF-EMFs, as indicated). The bar are means \pm S.E.M., $n=10$. * $p<0.05$. In panel F, the graph collects the mean values of the basal intracellular calcium concentration measured in undifferentiated or differentiated cell populations after 7-day exposure to turned off solenoid (C), 0.1 or 1.0 mT ELF-EMFs, as indicated. The bar are means \pm S.E.M., $n=5$. * $p<0.05$.



creasing ROS levels (Fig. 4A). Quantitative analyses demonstrated that in the undifferentiated PC12 phenotype, there was an increase, markedly in 1.0 mT exposed cells, in the number of cells showing a rise in ROS levels (Fig. 4B). Conversely, the NGF-differentiated PC12 cells were not influenced by the presence of ELF-EMFs (Fig. 4B).

Considering these results, we measured the specific activity of catalase, one enzyme of the detoxifying cell system.

The catalase activity resulted significantly reduced in cell extracts from undifferentiated cells exposed to 0.1 or 1.0 mT ELF-EMFs for 30 min (Fig. 4C). Conversely an increased activity resulted in cell extracts from chronic (7 days) exposure to 1.0 mT ELF-EMFs (Fig. 4D). The enzymatic activity assayed in differentiated cells appeared unchanged in all tested experimental conditions (Fig. 4C and D).

Intracellular calcium variations

To test the sensitivity of Ca^{2+} signalling dynamics to ELF-EMFs, intracellular Ca^{2+} levels in undifferentiated and neuron-like PC12 cells were monitored over 30 min in the presence of 0, 0.1 or 1.0 mT ELF-EMFs. The cell temporal analyses showed in Figure 5 represent PC12 spontaneous intracellular Ca^{2+} variations characterized by different temporal kinetics. In detail, undifferentiated cells

presented in equal amount a cell percentage showing no significant variation, or isolated single spikes or instable Ca^{2+} levels (Fig. 5A-C); neuronal-like PC12 cells showed or no significant variation, or isolated single spikes or high frequency Ca^{2+} waves (Fig. 5A, B and D). Quantitative analyses of these results revealed that the presence of ELF-EMFs seemed to significantly reduce only the spontaneous activity of undifferentiated PC12 cells, decreasing the number of active cells (Fig. 5E). These differences were not observed when the cells were exposed for 7 days (data not shown). The analysis of the basal intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) revealed that the mean value of $[\text{Ca}^{2+}]_i$ did not significantly change during acute (30 min) exposure, while increased in undifferentiated cells after 7 day exposure to ELF-EMFs becoming significantly higher in 1.0 mT-exposed cells in respect to control ones (Fig. 5F).

When PC12 cells were stimulated by a depolarizing agent (50 mM KCl), an increase of intracellular Ca^{2+} level was observed (graph in Fig. 6). This cellular response was not affected by the presence of ELF-EMFs because it was developed in control and exposed cells at the same amount (Table in Fig. 6).

When the same stimulus was applied in Ca^{2+} -free medium, another Ca^{2+} rise could be observed, followed by a further ionic release from internal stores due to the addition of thapsigargin (Tg), an inhibitor of internal store

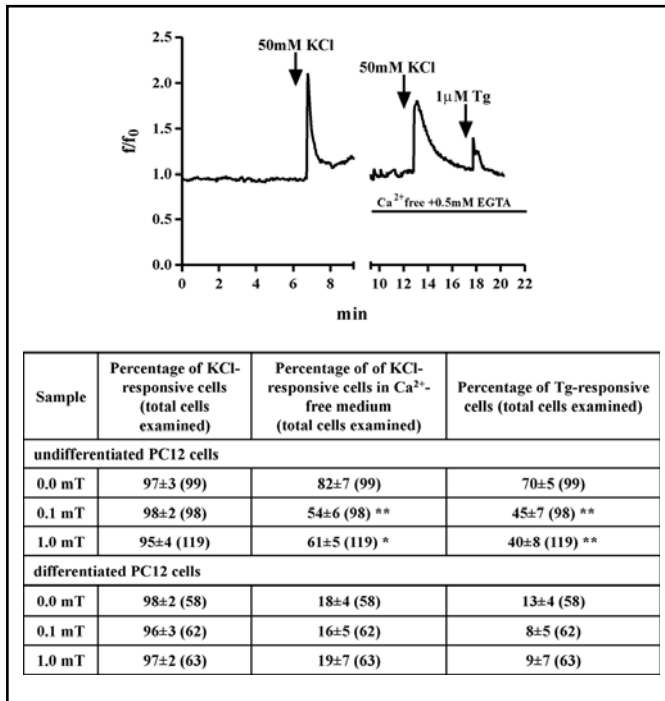


Fig. 6. Intracellular calcium variations induced by KCl and thapsigargin (Tg) in PC12 cells in presence or absence (Ca²⁺-free medium+0.5 mM EGTA) of external Ca²⁺. The graph is representative of a single cell behaviour. For each experimental condition (undifferentiated or differentiated PC12 cells, non-exposed or exposed cells to 0.1 or 1.0 mT ELF-EMFs), at least 10 fields (57-63 number of cells) in different coverslips were analysed. The table shows the quantitative analyses of Fluo4-loaded undifferentiated and neuronal-like PC12 cells responsive to extracellularly administered stimuli. The data reported are mean ± S.E.M., n=10. * p<0.01 and ** p<0.005 vs 0.0 mT exposed control.

Ca²⁺/ATPases [40]. In this condition, a great number of undifferentiated PC12 cells were responsive to KCl and Tg (82 ± 7% and 70 ± 5%, respectively). In presence of ELF-EMFs (0.1 or 1.0 mT) the responsive cells were significantly reduced. The NGF-differentiated PC12 cells were less sensitive to KCl and Tg in Ca²⁺-free medium and the presence of ELF-EMFs did not change this behaviour (Table in Fig. 6).

To monitor membrane potential during cell exposure to ELF-EMFs, PC12 phenotypes, undifferentiated and NGF-differentiated one, were also loaded with DiBAC₄ probe and observed with the confocal microscope equipped with Helmholtz coils. During 30 min observation, both PC12 phenotypes exhibited stable membrane potential in about the 85 ± 7% of the undifferentiated or 85 ± 9% of the NGF-differentiated cell population and this condition did not significantly change in presence of

0.1 mT (90 ± 9 and 89 ± 8% for undifferentiated and differentiated cells respectively) or 1.0 mT (92 ± 7% and 96 ± 4% for undifferentiated and differentiated cells respectively) ELF-EMFs.

Discussion

Nowadays, the enormous benefits of using electricity in our everyday life and in health care are unquestionable, but the presence of possible inappropriate effects, or health consequences, needs to be clearly identified. The results of research currently available have often been contradictory, and this adds to the confusion and the lack of confidence that consistent conclusions regarding safety can be provided.

Since the 90s the variety of biological effects induced by the exposure to ELF-EMFs were known and recently, Foster reviewed the proposed mechanisms of interaction of ELF-EMFs and biological system [11] but the conclusive theories are far to be reached. This also depend on the extreme variability of the biological models studied, and on the lack of a standardization of protocols of ELF-EMF generation and exposure.

The results of our experiments show a slight transient decrease in the proliferation rate and in morphological differentiation after 1.0 mT ELF-EMFs exposure. The lack of significant effect induced by ELF-EMFs, was also supported by the fact that GAP43 and MAP2, neuronal cytoskeletal markers, levels appeared similar in exposed cells to those of control ones. These results are apparently in contrast with the data already published [6, 21, 24, 42] it should be considered that different ELF-EMF intensities and cell models (even if of the same phenotype) were reported.

The effects of ELF-EMFs on cell biology are often dependent on the cell model, rather than on a direct action of the ELF-EMFs on some specific steps in the progression of the cell cycle or differentiation.

As previously demonstrated by Ross SM, markedly different effects, that can range from inhibition to stimulation of cell proliferation, can be obtained, depending on the signal parameters (amplitude and frequency of the ELF-EMFs) and the types of cell substrate used [43]. Similarly, the genotoxic potential effects of ELF-EMFs reflect differences among the cellular targets examined [12].

This is also supported by results on the effects of ELF-EMFs on stromal stem cell proliferation (CFU-f: colony forming units-fibroblasts); indeed, exposed CFU-

f cells from female mice showed a reduction, while CFU-f cells from male mice showed no decrease in cell proliferation [44].

Biological effects induced by different modalities of ELF-EMF exposures on cell lines, have been frequently noted, but the basic interaction mechanism(s) between the field exposure and living matter remain unknown [11, 45]. Even if some hypotheses have been suggested, none of them is adequately supported by experimental data [13, 46]. Various cellular components, processes, and systems can be affected by exposure to ELF-EMFs, e.g. the cell membranes (both internal and external) and signal transduction pathways [47], cell-cycle regulation and cell proliferation and/or differentiation [4, 29]. Also, direct or indirect DNA damage has been measured in different substrates, although this does not lead directly to genotoxic effects [48-50].

Different physiological pathways have been measured in several laboratories using different substrates and have shown the presence of biological effects (acute and chronic) derived from ELF-EMF exposure, even if not without troublesome questions, and these responses can be associated with detectable changes in cell physiology. This last consideration suggests that there could exist a compensatory mechanisms at the intracellular signalling, translational or/and posttranslational levels.

For this reason, a possible common initial cellular event, affected by ELF-EMF exposure, could be hypothesised. Based on an extensive review of the literature, Simko and Mattsson [29] recently suggested that an increase of the levels of free radicals could be the “transducer” of ELF-EMFs-induced effects. Free radicals are intermediates in natural processes and they could be the stimulus to induce an “activated state” of the cell that then enhances the release of free radicals, in turn leading to biological or pathological events [51].

Wolf and Colleagues showed that ELF-EMFs effects were prevented pre-treating the cells with antioxidants, suggesting that redox reactions were involved. Accordingly, after exposure to 1.0 mT ELF-EMFs, the cells showed a significant increase in ROS accumulation that was decreased by the addition to the culture medium of an adequate scavenger [10]. Also, under our experimental conditions, it was possible to note a significant modification in ROS production in undifferentiated PC12 cells while the differentiated phenotype seemed not to be affected. Moreover, *in vivo* experiments also appear to confirm that chronic exposure to ELF-EMFs can generate an oxidative stress status [31, 52-61]. In addition, the presence of a “stress” status is also demonstrated by the

fact that the enzymatic activity of catalase, involved in the catalytic removal of ROS accumulated as a possible effect of ELF-EMF exposure, was decreased during chronic exposure. Conversely, its increased activity after chronic exposure could reflect that absence of ROS accumulation and a possible adaptation cell response to the presence of ELF-EMFs.

The *in vitro* experiments reported by [62-64] in rodents, show that exposure to ELF-EMFs can induce variable and species-specific alterations in the oxidative stress pathway.

If ROS generation and accumulation can be considered the first cellular event of ELF-EMFs exposure, the modification of intracellular Ca^{2+} levels could be one of the most important mechanisms by which ROS have their multiple actions in cells [65]. Over the past few years, a lot of data showed that ELF-EMFs exposure can induce modifications of intracellular Ca^{2+} levels that can be related to different physiological mechanisms: i.e. differentiation of chromaffin cells into neuronal-like cells [21]; cell death by apoptosis induced by several agents in different human cell systems [66]; and functional modifications of cells of the immune system [67, 68]. All of these effects, and many more others, can be attributed to modifications to the Ca^{2+} homeostasis by ELF-EMF exposure across different mechanisms, such as the involvement of P2Y membrane receptors [69]; the activation of mechanically operated stretch-activated Ca^{2+} channels [70]; and the enhancement of the expression of voltage-gated Ca^{2+} channels [20]. All of these pathways include, at least in part, the ionic transmembrane channels that represent the main pathways for cell communication, and particularly in excitable cells.

Anyway, cells have several Ca^{2+} -mobilizing messengers that transduce a large variety of extracellular stimuli for the regulation of various intracellular Ca^{2+} -sensitive targets [71].

In undifferentiated PC12 cells, during 30 min cell exposure the increase of ROS production was accompanied by a decrease of spontaneous intracellular Ca^{2+} variations as well as a decrease to KCl and Tg stimulation in external Ca^{2+} -free medium, while the responses to depolarizing KCl in normal external medium and membrane potential remained unchanged.

As reported from several studies, a close relationship between intracellular Ca^{2+} changes and increases in ROS production exists under different experimental conditions. For example: (i) in cardiac tissue, a cytosolic, and subsequently mitochondrial, Ca^{2+} overload impaired mitochondrial bioenergetics [61]; and (ii) in neurons, tran-

sient deprivation of oxygen and glucose during temporary ischemia coupled with elevation in $[Ca^{2+}]_i$ triggered ROS generation and mitochondrial permeabilization, resulting in neural cell death [72]. There after, also in our model, some aspects of stimulated Ca^{2+} -dependent activities and spontaneous spike generation also appeared to be influenced by the exposure to ELF-EMFs.

In addition, spontaneous activity and the responses in Ca^{2+} -free medium have a common key that is Ca^{2+} channels present on the intracellular stores, possible targets of ROS generated by the presence of ELF-EMFs. The importance of this observation comes out if one considers that intracellular Ca^{2+} channel status is strictly dependent on the redox molecules present into the cells [73].

The results reported so far do not demonstrate drastic effects arising from the exposure to ELF-EMFs on the proliferative ability or the differentiation of the PC12 cells, although there were transitory modifications which were significant with respect to the cells that were not exposed. This leads to the hypothesis that the cells were not completely insensitive to the exposure to the field, but they were instead able to overcome a condition of stress.

The cell responses to external stimuli also include the activation of various cytoplasmatic systems such as the oxidative stress machinery and those regulating intracellular calcium. In addition, as mentioned above, these

pathways were described to be involved in ELF-EMFs in other experimental models [27, 68, 74-78].

In addition, thanks to the use of the Helmholtz coils adapted to a confocal microscope, the innovative experimental approach of this study remains the possibility to monitor cell responses and functionality during the ELF-EMFs exposures.

In conclusion, the data reported showed that undifferentiated and differentiating PC12 cells appeared more vulnerable to ELF-EMFs exposure while the fully differentiated phenotype was less sensitive and more stable.

Even if further studies remain necessary to identify the ROS produced by this exposure to ELF-EMFs and the class of the calcium channels involved in the development of ELF-EMFs interaction, we propose this biochemical substrate as the candidate for the cellular “*primum movens*” of ELF-EMF-induced effects on biological systems.

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References

- Vecchia P: Exposure of humans to electromagnetic fields. Standards and regulations. *Ann Ist Super Sanita* 2007;43:260-267.
- Berg A, Berg H: Influence of ELF sinusoidal electromagnetic fields on proliferation and metabolite yield of fungi. *Electromagn Biol Med* 2006;25:71-77.
- Crumpton MJ, Collins AR: Are environmental electromagnetic fields genotoxic? *DNA Repair (Amst)* 2004;3:1385-1387.
- Frahm J, Lantow M, Lupke M, Weiss DG, Simko M: Alteration in cellular functions in mouse macrophages after exposure to 50 Hz magnetic fields. *J Cell Biochem* 2006;99:168-177.
- Gartzke J, Lange K: Cellular target of weak magnetic fields: ionic conduction along actin filaments of microvilli. *Am J Physiol Cell Physiol* 2002;283:C1333-1346.
- Lisi A, Foletti A, Ledda M, Rosola E, Giuliani L, D’Emilia E, Grimaldi S: Extremely low frequency 7 Hz 100 microT electromagnetic radiation promotes differentiation in the human epithelial cell line HaCaT. *Electromagn Biol Med* 2006;25:269-280.
- Palumbo R, Capasso D, Brescia F, Mita P, Sarti M, Bersani F, Scarfi MR: Effects on apoptosis and reactive oxygen species formation by Jurkat cells exposed to 50 Hz electromagnetic fields. *Bioelectromagnetics* 2006;27:159-162.
- Santini MT, Ferrante A, Rainaldi G, Indovina P, Indovina PL: Extremely low frequency (ELF) magnetic fields and apoptosis: a review. *Int J Radiat Biol* 2005;81:1-11.
- Vaughan TE, Weaver JC: Molecular change signal-to-noise criteria for interpreting experiments involving exposure of biological systems to weakly interacting electromagnetic fields. *Bioelectromagnetics* 2005;26:305-322.
- Wolf FI, Torsello A, Tedesco B, Fasanella S, Boninsegna A, D’Ascenzo M, Grassi C, Azzena GB, Cittadini A: 50-Hz extremely low frequency electromagnetic fields enhance cell proliferation and DNA damage: possible involvement of a redox mechanism. *Biochim Biophys Acta* 2005;1743:120-129.
- Foster KR: Mechanisms of interaction of extremely low frequency electric fields and biological systems. *Radiat Prot Dosimetry* 2003;106:301-310.
- Ivancsits S, Pilger A, Diem E, Jahn O, Rudiger HW: Cell type-specific genotoxic effects of intermittent extremely low-frequency electromagnetic fields. *Mutat Res* 2005;583:184-188.
- Repacholi MH, Greenebaum B: Interaction of static and extremely low frequency electric and magnetic fields with living systems: health effects and research needs. *Bioelectromagnetics* 1999;20:133-160.

- 14 Guler G, Turkozer Z, Ozgur E, Seyhan N: Antioxidants alleviate electric field-induced effects on lung tissue based on assays of heme oxygenase-1, protein carbonyl content, malondialdehyde, nitric oxide, and hydroxyproline. *Sci Total Environ* 2009;407:1326-1332.
- 15 Guler G, Turkozer Z, Ozgur E, Tomruk A, Seyhan N, Karasu C: Protein oxidation under extremely low frequency electric field in guinea pigs. Effect of N-acetyl-L-cysteine treatment. *Gen Physiol Biophys* 2009;28:47-55.
- 16 Guler G, Turkozer Z, Tomruk A, Seyhan N: The protective effects of N-acetyl-L-cysteine and epigallocatechin-3-gallate on electric field-induced hepatic oxidative stress. *Int J Radiat Biol* 2008;84:669-680.
- 17 Singh N, Lai H: 60 Hz magnetic field exposure induces DNA crosslinks in rat brain cells. *Mutat Res* 1998;400:313-320.
- 18 Mathie A, Kennard LE, Veale EL: Neuronal ion channels and their sensitivity to extremely low frequency weak electric field effects. *Radiat Prot Dosimetry* 2003;106:311-316.
- 19 Sieron A, Labus L, Nowak P, Cieslar G, Brus H, Durczok A, Zagzil T, Koszrzewa RM, Brus R: Alternating extremely low frequency magnetic field increases turnover of dopamine and serotonin in rat frontal cortex. *Bioelectromagnetics* 2004;25:426-430.
- 20 Grassi C, D'Ascenzo M, Torsello A, Martinotti G, Wolf F, Cittadini A, Azzena GB: Effects of 50 Hz electromagnetic fields on voltage-gated Ca²⁺ channels and their role in modulation of neuroendocrine cell proliferation and death. *Cell Calcium* 2004;35:307-315.
- 21 Morgado-Valle C, Verdugo-Diaz L, Garcia DE, Morales-Orozco C, Drucker-Colin R: The role of voltage-gated Ca²⁺ channels in neurite growth of cultured chromaffin cells induced by extremely low frequency (ELF) magnetic field stimulation. *Cell Tissue Res* 1998;291:217-230.
- 22 Piacentini R, Ripoli C, Mezzogori D, Azzena GB, Grassi C: Extremely low-frequency electromagnetic fields promote in vitro neurogenesis via upregulation of Ca(v)1-channel activity. *J Cell Physiol* 2008;215:129-139.
- 23 Blackman CF, Benane SG, House DE, Pollock MM: Action of 50 Hz magnetic fields on neurite outgrowth in pheochromocytoma cells. *Bioelectromagnetics* 1993;14:273-286.
- 24 McFarlane EH, Dawe GS, Marks M, Campbell IC: Changes in neurite outgrowth but not in cell division induced by low EMF exposure: influence of field strength and culture conditions on responses in rat PC12 pheochromocytoma cells. *Bioelectrochemistry* 2000;52:23-28.
- 25 Liboff AR, Cherng S, Jenrow KA, Bull A: Calmodulin-dependent cyclic nucleotide phosphodiesterase activity is altered by 20 microT magnetostatic fields. *Bioelectromagnetics* 2003;24:32-38.
- 26 Manikonda PK, Rajendra P, Devendranath D, Gunasekaran B, Channakeshava, Aradhya RS, Sashidhar RB, Subramanyam C: Influence of extremely low frequency magnetic fields on Ca²⁺ signaling and NMDA receptor functions in rat hippocampus. *Neurosci Lett* 2007;413:145-149.
- 27 Morabito C, Rovetta F, Bizzarri M, Mazzoleni G, Fanò G, Mariggiò MA: Modulation of redox status and calcium handling by extremely low frequency electromagnetic fields in C2C12 muscle cells: A real-time, single-cell approach. *Free Radic Biol Med* 2010;48:579-589.
- 28 Vyklicky L Jr: Calcium-mediated modulation of N-methyl-D-aspartate (NMDA) responses in cultured rat hippocampal neurones. *J Physiol* 1993;470:575-600.
- 29 Simko M, Mattsson MO: Extremely low frequency electromagnetic fields as effectors of cellular responses in vitro: possible immune cell activation. *J Cell Biochem* 2004;93:83-92.
- 30 Di Loreto S, Falone S, Caracciolo V, Sebastiani P, D'Alessandro A, Mirabilio A, Zimmitti V, Amicarelli F: Fifty hertz extremely low-frequency magnetic field exposure elicits redox and trophic response in rat-cortical neurons. *J Cell Physiol* 2009;219:334-343.
- 31 Regoli F, Gorbi S, Machella N, Tedesco S, Benedetti M, Bocchetti R, Notti A, Fattorini D, Piva F, Principato G: Prooxidant effects of extremely low frequency electromagnetic fields in the land snail *Helix aspersa*. *Free Radic Biol Med* 2005;39:1620-1628.
- 32 Zwirska-Korczała K, Jochem J, Adamczyk-Sowa M, Sowa P, Polaniak R, Birkner E, Latocha M, Pilc K, Suchanek R: Effect of extremely low frequency of electromagnetic fields on cell proliferation, antioxidative enzyme activities and lipid peroxidation in 3T3-L1 preadipocytes-an in vitro study. *J Physiol Pharmacol* 2005;56 Suppl 6:101-108.
- 33 Farina M, Farina M, Mariggiò MA, Pietrangelo T, Stupak JJ, Morini A, Fanò G: ELF-EMFs induced effects on cell lines: controlling elf generation in laboratory. *Progr Electromagn Res B* 2010;24:131-153.
- 34 Greene LA, Tischler AS: Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci U S A* 1976;73:2424-2428.
- 35 Guarnieri S, Pilla R, Morabito C, Sacchetti S, Mancinelli R, Fanò G, Mariggiò MA: Extracellular guanosine and GTP promote expression of differentiation markers and induce S-phase cell-cycle arrest in human SH-SY5Y neuroblastoma cells. *Int J Dev Neurosci* 2009;27:135-147.
- 36 Gysbers JW, Guarnieri S, Mariggiò MA, Pietrangelo T, Fanò G, Rathbone MP: Extracellular guanosine 5' triphosphate enhances nerve growth factor-induced neurite outgrowth via increases in intracellular calcium. *Neuroscience* 2000;96:817-824.
- 37 Gunning PW, Landreth GE, Bothwell MA, Shooter EM: Differential and synergistic actions of nerve growth factor and cyclic AMP in PC12 cells. *J Cell Biol* 1981;89:240-245.
- 38 Gysbers JW, Rathbone MP: Neurite outgrowth in PC12 cells is enhanced by guanosine through both cAMP-dependent and -independent mechanisms. *Neurosci Lett* 1996;220:175-178.
- 39 Gryniewicz G, Poenie M, Tsien RY: A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440-3450.
- 40 Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP: Thapsigargin, a tumor promoter, discharges intracellular Ca²⁺ stores by specific inhibition of the endoplasmic reticulum Ca²⁺-ATPase. *Proc Natl Acad Sci U S A* 1990;87:2466-2470.
- 41 Lacy-Hulbert A, Metcalfe JC, Hesketh R: Biological responses to electromagnetic fields. *Faseb J* 1998;12:395-420.
- 42 Zhang L, Zhou L, Vega-Gonzalez A, Mendoza D, Drucker-Colin R: Extremely low frequency magnetic fields promote neurite varicosity formation and cell excitability in cultured rat chromaffin cells. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1997;118:295-299.
- 43 Ross SM: Combined DC and ELF magnetic fields can alter cell proliferation. *Bioelectromagnetics* 1990;11:27-36.
- 44 Van Den Heuvel R, Leppens H, Nemethova G, Verschaevae L: Haemopoietic cell proliferation in murine bone marrow cells exposed to extreme low frequency (ELF) electromagnetic fields. *Toxicol In Vitro* 2001;15:351-355.
- 45 Kavet R, Stuchly MA, Bailey WH, Bracken TD: Evaluation of biological effects, dosimetric models, and exposure assessment related to ELF electric- and magnetic-field guidelines. *Appl Occup Environ Hyg* 2001;16:1118-1138.
- 46 Preece AW, Hand JW, Clarke RN, Stewart A: Power frequency electromagnetic fields and health. Where's the evidence? *Phys Med Biol* 2000;45:R139-154.

- 47 Czyn J, Nikolova T, Schuderer J, Kuster N, Wobus AM: Non-thermal effects of power-line magnetic fields (50 Hz) on gene expression levels of pluripotent embryonic stem cells—the role of tumour suppressor p53. *Mutat Res* 2004;557:63-74.
- 48 Fatigoni C, Dominici L, Moretti M, Villarini M, Monarca S: Genotoxic effects of extremely low frequency (ELF) magnetic fields (MF) evaluated by the Tradescantia-micronucleus assay. *Environ Toxicol* 2005;20:585-591.
- 49 Stronati L, Testa A, Villani P, Marino C, Lovisolo GA, Conti D, Russo F, Fresegha AM, Cordelli E: Absence of genotoxicity in human blood cells exposed to 50 Hz magnetic fields as assessed by comet assay, chromosome aberration, micronucleus, and sister chromatid exchange analyses. *Bioelectromagnetics* 2004;25:41-48.
- 50 Villarini M, Moretti M, Scassellati-Sforzolini G, Boccioli B, Pasquini R: Effects of co-exposure to extremely low frequency (50 Hz) magnetic fields and xenobiotics determined in vitro by the alkaline comet assay. *Sci Total Environ* 2006;361:208-219.
- 51 Belia S, Santilli F, Beccafico S, De Feudis L, Morabito C, Davi G, Fanò G, Mariggiò MA: Oxidative-induced membrane damage in diabetes lymphocytes: effects on intracellular Ca²⁺ homeostasis. *Free Radic Res* 2009;43:138-148.
- 52 Harakawa S, Inoue N, Hori T, Tochio K, Kariya T, Takahashi K, Doge F, Martin DE, Saito A, Suzuki H, Nagasawa H: Effects of exposure to a 50 Hz electric field on plasma levels of lactate, glucose, free fatty acids, triglycerides and creatine phosphokinase activity in hind-limb ischemic rats. *J Vet Med Sci* 2005;67:969-974.
- 53 Yokus B, Cakir DU, Akdag MZ, Sert C, Mete N: Oxidative DNA damage in rats exposed to extremely low frequency electro magnetic fields. *Free Radic Res* 2005;39:317-323.
- 54 Canseven AG, Coskun S, Seyhan N: Effects of various extremely low frequency magnetic fields on the free radical processes, natural antioxidant system and respiratory burst system activities in the heart and liver tissues. *Indian J Biochem Biophys* 2008;45:326-331.
- 55 Coskun S, Balabanli B, Canseven A, Seyhan N: Effects of continuous and intermittent magnetic fields on oxidative parameters in vivo. *Neurochem Res* 2009;34:238-243.
- 56 Guler G, Seyhan N, Aricioglu A: Effects of static and 50 Hz alternating electric fields on superoxide dismutase activity and TBARS levels in guinea pigs. *Gen Physiol Biophys* 2006;25:177-193.
- 57 Guler G, Turkozer Z, Seyhan N: Electric field effects on Guinea pig serum: the role of free radicals. *Electromagn Biol Med* 2007;26:207-223.
- 58 Seyhan N, Canseven AG: In vivo effects of ELF MFs on collagen synthesis, free radical processes, natural antioxidant system, respiratory burst system, immune system activities, and electrolytes in the skin, plasma, spleen, lung, kidney, and brain tissue of guinea pigs. *Electromagn Biol Med* 2006;25:291-305.
- 59 Seyhan N, Guler G: Review of in vivo static and ELF electric fields studies performed at Gazi Biophysics Department. *Electromagn Biol Med* 2006;25:307-323.
- 60 Turkozer Z, Guler G, Seyhan N: Effects of exposure to 50 Hz electric field at different strengths on oxidative stress and antioxidant enzyme activities in the brain tissue of guinea pigs. *Int J Radiat Biol* 2008;84:581-590.
- 61 Solaini G, Harris DA: Biochemical dysfunction in heart mitochondria exposed to ischaemia and reperfusion. *Biochem J* 2005;390:377-394.
- 62 Falone S, Mirabilio A, Carbone MC, Zimmitti V, Di Loreto S, Mariggiò MA, Mancinelli R, Di Ilio C, Amicarelli F: Chronic exposure to 50Hz magnetic fields causes a significant weakening of antioxidant defence systems in aged rat brain. *Int J Biochem Cell Biol* 2008;40:2762-2770.
- 63 Lee JS, Ahn SS, Jung KC, Kim YW, Lee SK: Effects of 60 Hz electromagnetic field exposure on testicular germ cell apoptosis in mice. *Asian J Androl* 2004;6:29-34.
- 64 Mariggiò MA, Amicarelli F, Grossi MR, Falone S, Morabito C, Tettamanti E, Fanò G, Cuccurullo F, Di Ilio C: Extremely low frequency electromagnetic fields and oxidative stress in excitable cell lines; in Kostarakis P (ed): *Biological Effects of Electromagnetic Fields*. Crete, 2006, pp 1043-1050.
- 65 Camello-Almaraz C, Gomez-Pinilla PJ, Pozo MJ, Camello PJ: Mitochondrial reactive oxygen species and Ca²⁺ signaling. *Am J Physiol Cell Physiol* 2006;291:C1082-1088.
- 66 Fanelli C, Coppola S, Barone R, Colussi C, Gualandi G, Volpe P, Ghibelli L: Magnetic fields increase cell survival by inhibiting apoptosis via modulation of Ca²⁺ influx. *Faseb J* 1999;13:95-102.
- 67 Walleczek J: Electromagnetic field effects on cells of the immune system: the role of calcium signaling. *Faseb J* 1992;6:3177-3185.
- 68 Walleczek J, Budinger TF: Pulsed magnetic field effects on calcium signaling in lymphocytes: dependence on cell status and field intensity. *FEBS Lett* 1992;314:351-355.
- 69 Takahashi K, Doge F, Yoshioka M: Prolonged Ca²⁺ transients in ATP-stimulated endothelial cells exposed to 50 Hz electric fields. *Cell Biol Int* 2005;29:237-243.
- 70 Sun S, Cho M: Human fibroblast migration in three-dimensional collagen gel in response to noninvasive electrical stimulus. II. Identification of electrocoupling molecular mechanisms. *Tissue Eng* 2004;10:1558-1565.
- 71 Berridge MJ, Lipp P, Bootman MD: The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 2000;1:11-21.
- 72 Brailoiu E, Churamani D, Pandey V, Brailoiu GC, Tuluc F, Patel S, Dun NJ: Messenger-specific role for nicotinic acid adenine dinucleotide phosphate in neuronal differentiation. *J Biol Chem* 2006;281:15923-15928.
- 73 Hidalgo C, Donoso P, Carrasco MA: The ryanodine receptors Ca²⁺ release channels: cellular redox sensors? *IUBMB Life* 2005;57:315-322.
- 74 Liburdy RP, Callahan DE, Harland J, Dunham E, Sloma TR, Yaswen P: Experimental evidence for 60 Hz magnetic fields operating through the signal transduction cascade. Effects on calcium influx and c-MYC mRNA induction. *FEBS Lett* 1993;334:301-308.
- 75 Lindstrom E, Lindstrom P, Berglund A, Lundgren E, Mild KH: Intracellular calcium oscillations in a T-cell line after exposure to extremely-low-frequency magnetic fields with variable frequencies and flux densities. *Bioelectromagnetics* 1995;16:41-47.
- 76 Loschinger M, Thumm S, Hammerle H, Rodemann HP: Induction of intracellular calcium oscillations in human skin fibroblast populations by sinusoidal extremely low-frequency magnetic fields (20 Hz, 8 mT) is dependent on the differentiation state of the single cell. *Radiat Res* 1999;151:195-200.
- 77 Mattsson MO, Lindstrom E, Still M, Lindstrom P, Mild KH, Lundgren E: [Ca²⁺]_i rise in Jurkat E6-1 cell lines from different sources as a response to 50 Hz magnetic field exposure as a reproducible effect and independent of poly-L-lysine treatment. *Cell Biol Int* 2001;25:901-907.
- 78 Simko M: Cell type specific redox status is responsible for diverse electromagnetic field effects. *Curr Med Chem* 2007;14:1141-1152.