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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* neuritogenesis 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 neuritogenesis 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 neuritogenesis). 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 system 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 network 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 cell 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 neuritogenic 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 find 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* neuritogenesis 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 ± 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°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×/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 ± 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% CO_2 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, 100µg/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% CO_2 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, 10^4 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 Burker 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 10^5 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 (±S.E.M.) were then determined for each treatment.

Videoimaging on single cell

ROS levels, Ca^{2+}signalling, membrane potential variations were evaluated in both undifferentiated and differentiated PC12 cells with H_2DCF-DA (dichlorodihydrofluorescein diacetate, Molecular Probes, Eugene, OR, USA), Fluo-4 AM (Molecular Probes) or DIBAC$_4$ (*bis*-(1,3 dibutylbarbituric acid) trimethine oxonol, Molecular Probes) respectively. PC12 cells (3.0×10^4 cells/cm^2) 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 $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes, pH 7.3) containing 10 µM H_2DCF-DA or 5

μM Fluo-4 AM or 500 nM $DIBAC_4$ 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 ± 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 $[Ca^{2+}]_i$

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 sulphynpirazone then collected in the quartz cuvette and analysed using LS-5B Perkin-Elmer spectrofluorimeter. The $[Ca^{2+}]_i$ was calculated using the formula: $[Ca^{2+}]_i$ = Kd (F-Fmin)/(Fmax-F), where Fmax and Fmin were the fluorescences recorded at the end of each experiment, after the addition of 1 μM Ionomicin and 5 mM EGTA respectively; Kd (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 μg/mL PMSF, 10 μg/mL leupeptin, 5 μg/mL pepstatin A, and 10 μg/mL benzamidine. After five min of vortexing, the samples were centrifuged at 10000 x 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% polyacrilamide gels (SDS-PAGE). The proteins were then transferred to polyvinyldifluoride 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).

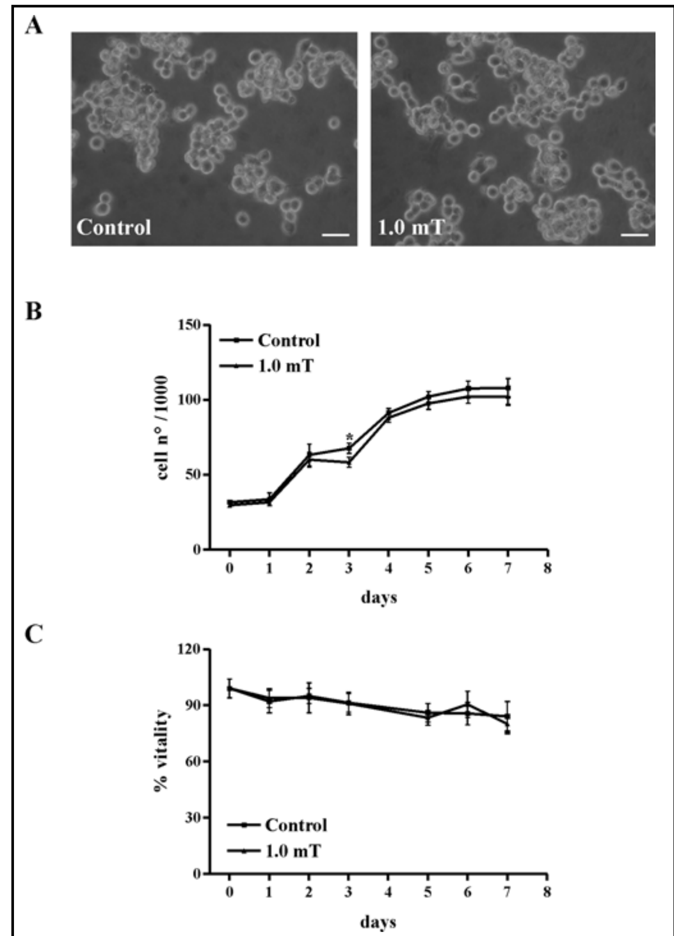
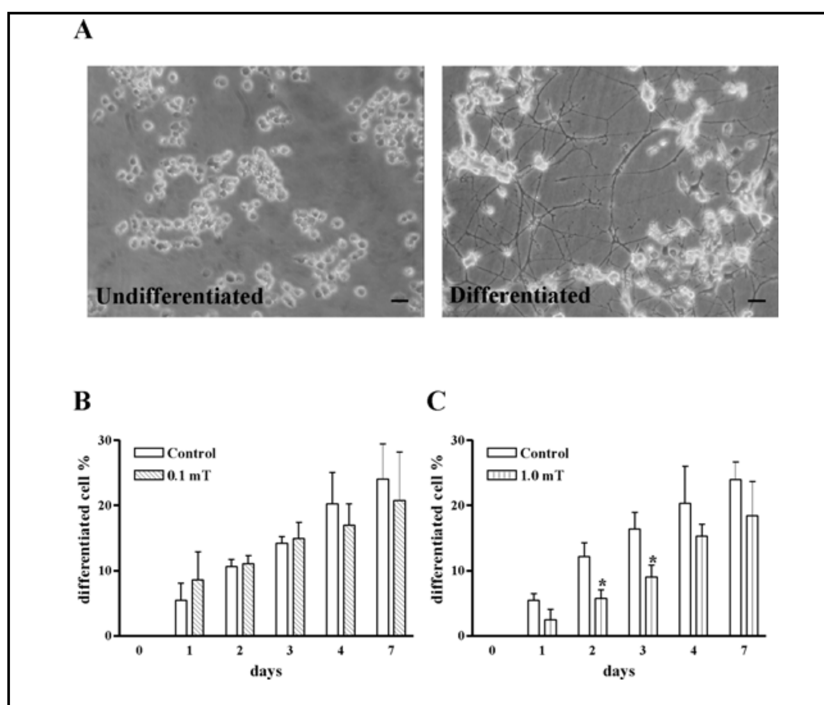


Fig. 1. PC12 cell morphology and proliferation. Panel A shows representative images of undifferentiated PC12 grown for 7 days in the absence (Control) or presence of 1.0 mT ELF-EMF, as indicated. Scale bar=20μm. Panel B and C report cell proliferation and viability respectively. Growth curves of PC12 cells grown in absence (Control) or presence of 1.0 mT ELF-EMFs, as indicated. The data are means ± 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 ± S.D., n = 8.

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=20µm. 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 ± S.E.M., n=10; * p<0.05.



containing 1 µg/ml pepstatin, 1 µg/ml leupeptin, and 100 µM 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 H_2O_2 consumption (ε= -0.04 mM^{-1} 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 H_2O_2, and 100 µg 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 solenoid.

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

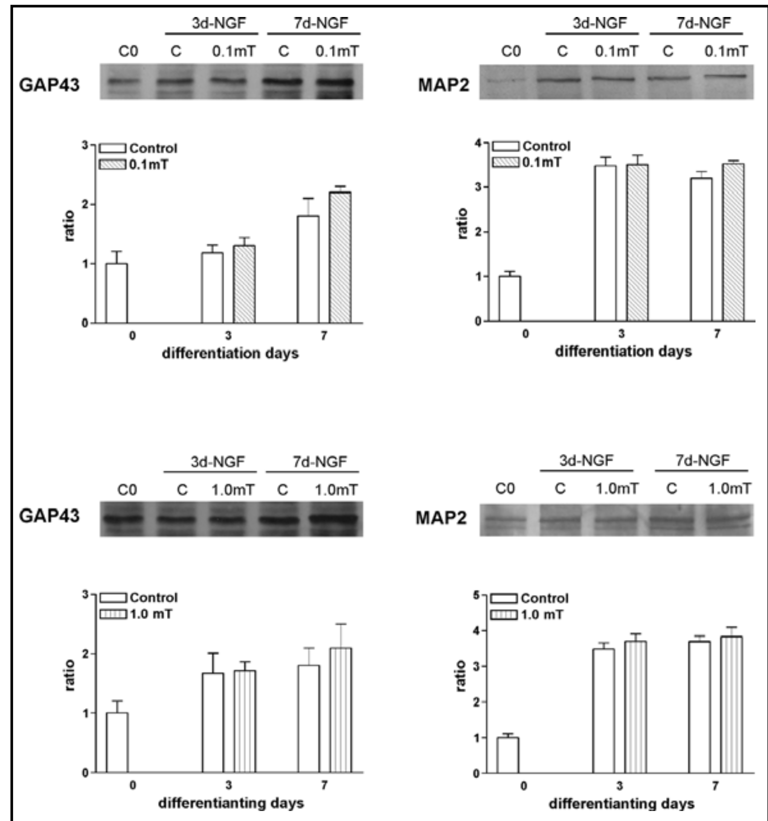


Fig. 3. GAP43 and MAP2 expression levels. Representative Western blots showing the levels of neuronal markers in differentiating PC12 cells incubated in absence (C) or in presence of 0.1 or 1.0 mT ELF-EMFs for 3 and 7 days (d), as indicated. The graphs collect densitometric analyses from three independent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

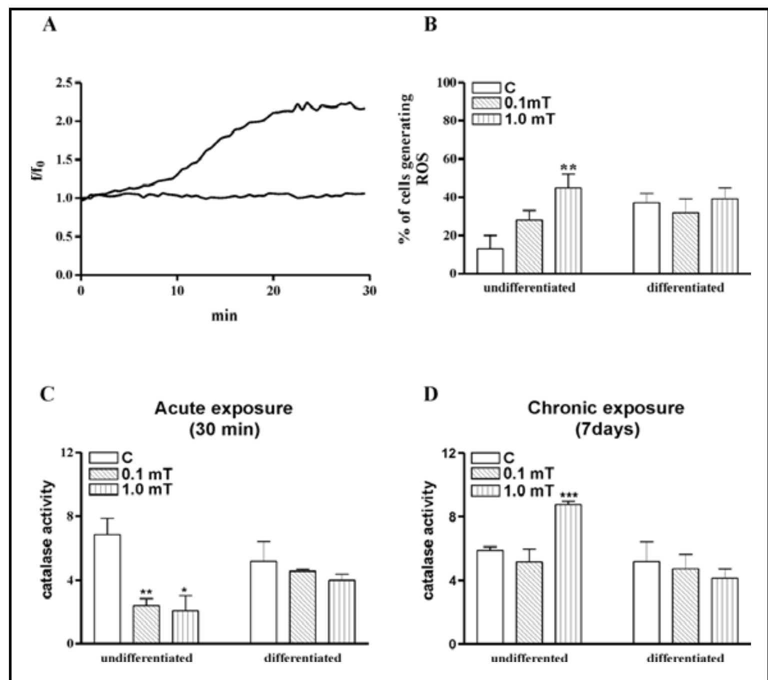


Fig. 4. Oxidative markers in PC12 cells. In panel A, the traces represent temporal analyses of the fluorescence (f/f_0) of single PC12 cells. Each trace is representative of a single cell behaviour. For each experimental condition (non-exposed or exposed cells to 0.1 or 1.0 mT ELF-EMFs), at least 10 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 ± S.E.M., n=10. ** $p<0.005$. In panels C and D, the specific activity of catalase, expressed as µmol/min/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 ± 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 H_2DCF-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-

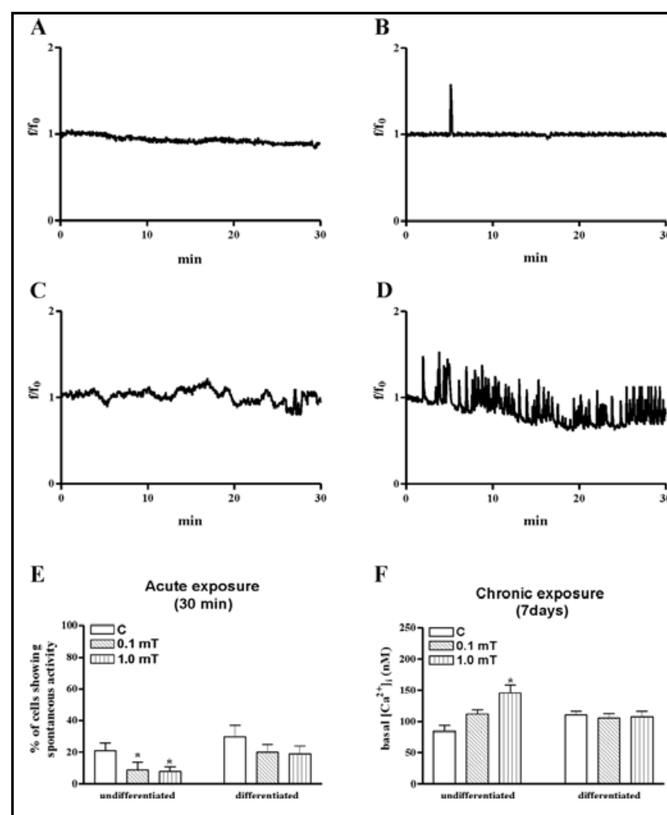


Fig. 5. Intracellular calcium. Panel A depicts no cellular activity; panel B, a single isolated intracellular Ca^{2+} spike; panel C, instable intracellular calcium levels; panel D, high frequency intracellular Ca^{2+} waves. In particular, undifferentiated PC12 cells showed in equal amount the A, B 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 ± 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 ± 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.

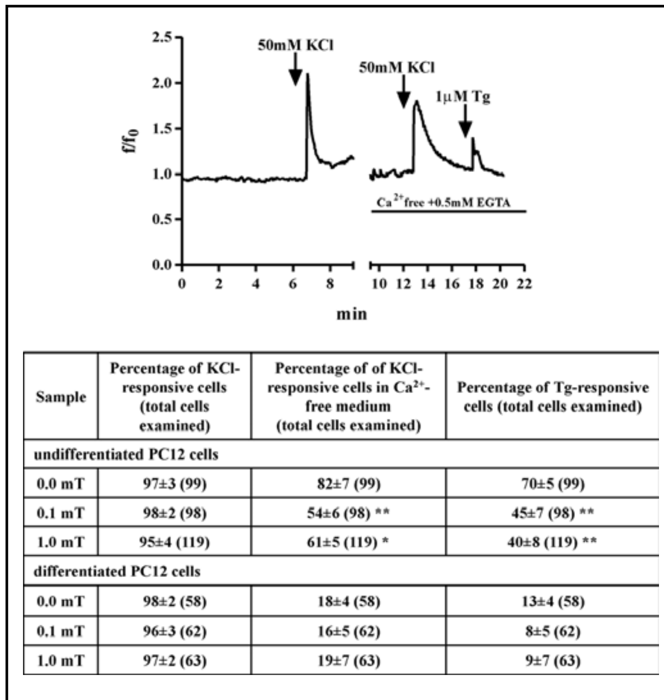
The catalase activity resulted significantly reduced in cell extracts from undifferentiated cells exposed to 0.1 or 1.0 mT ELF-EMFs for 30 min (Fig. 4C). Conversely an increased activity resulted in cell extracts from chronic (7 days) exposure to 1.0 mT ELF-EMFs (Fig. 4D). The enzymatic activity assayed 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 ($[Ca^{2+}]_i$) revealed that the mean value of $[Ca^{2+}]_i$ 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 responce 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



Sample	Percentage of KCl-responsive cells (total cells examined)	Percentage of of KCl-responsive cells in Ca^{2+}-free medium (total cells examined)	Percentage of Tg-responsive cells (total cells examined)
undifferentiated PC12 cells			
0.0 mT	97±3 (99)	82±7 (99)	70±5 (99)
0.1 mT	98±2 (98)	54±6 (98) **	45±7 (98) **
1.0 mT	95±4 (119)	61±5 (119) *	40±8 (119) **
differentiated PC12 cells			
0.0 mT	98±2 (58)	18±4 (58)	13±4 (58)
0.1 mT	96±3 (62)	16±5 (62)	8±5 (62)
1.0 mT	97±2 (63)	19±7 (63)	9±7 (63)

Fig. 6. Intracellular calcium variations induced by KCl and thapsigargin (Tg) in PC12 cells in presence or absence (Ca^{2+}-free medium+0.5 mM EGTA) of external Ca^{2+}. 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.

Ca^{2+}/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 Ca^{2+}-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 $DiBAC_4$ 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 cytoscheletal 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 Collegues 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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