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50-Hz extremely low frequency electromagnetic fields enhance cell proliferation and DNA damage: Possible involvement of a redox mechanism

Federica I. Wolf[a,*], Angela Torsello[a], Beatrice Tedesco[a], Silvia Fasanella[a], Alma Boninsegna[a], Marcello D'Ascenzo[b], Claudio Grassi[b], Gian Battista Azzena[b], Achille Cittadini[a]

[a]*Institute of General Pathology and Giovanni XXIII Cancer Research Center, L.go F. Vito, 1-00168 Rome, Italy*
[b]*Institute of Human Physiology, Catholic University of Sacred Heart, Faculty of Medicine, L.go F. Vito, 1-00168 Rome, Italy*

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Abstract

HL-60 leukemia cells, Rat-1 fibroblasts and WI-38 diploid fibroblasts were exposed for 24–72 h to 0.5–1.0-mT 50-Hz extremely low frequency electromagnetic field (ELF-EMF). This treatment induced a dose-dependent increase in the proliferation rate of all cell types, namely about 30% increase of cell proliferation after 72-h exposure to 1.0 mT. This was accompanied by increased percentage of cells in the S-phase after 12- and 48-h exposure. The ability of ELF-EMF to induce DNA damage was also investigated by measuring DNA strand breaks. A dose-dependent increase in DNA damage was observed in all cell lines, with two peaks occurring at 24 and 72 h. A similar pattern of DNA damage was observed by measuring formation of 8-OHdG adducts. The effects of ELF-EMF on cell proliferation and DNA damage were prevented by pretreatment of cells with an antioxidant like α-tocopherol, suggesting that redox reactions were involved. Accordingly, Rat-1 fibroblasts that had been exposed to ELF-EMF for 3 or 24 h exhibited a significant increase in dichlorofluorescein-detectable reactive oxygen species, which was blunted by α-tocopherol pretreatment. Cells exposed to ELF-EMF and examined as early as 6 h after treatment initiation also exhibited modifications of NFκB-related proteins (p65-p50 and IκBα), which were suggestive of increased formation of p65-p50 or p65-p65 active forms, a process usually attributed to redox reactions. These results suggest that ELF-EMF influence proliferation and DNA damage in both normal and tumor cells through the action of free radical species. This information may be of value for appraising the pathophysiologic consequences of an exposure to ELF-EMF.

Keywords: 8-OHdG; Single strand break; Cell cycle; DCF; NFκB; IκB; α-Tocopherol

1. Introduction

Environmental exposure to extremely low frequency (ELF) electromagnetic fields (EMF) is strongly increased in developed countries as a consequence of the distribution and use of electricity.

Since Wertheimer and Leeper [1] argued that the frequency of childhood cancer correlated to the electrical wiring configuration running nearby their houses, the correlation between ELF-EMF exposure and cancer risk has become a matter of public concern. As a consequence, the possible effects of ELF-EMF on biological systems were extensively investigated. Some epidemiological studies offered positive evidence for a correlation between exposure to ELF-EMF and increased incidence of brain, breast and hematological malignancies, but these results have not been confirmed in other studies [2–5]. Studies with laboratory animals similarly produced inconclusive or contradictory results; the small number of animals examined and the lack of properly standardised exposure parameters may have contributed to such negative or

* Corresponding author. Federica I. Wolf Institute of General Pathology, Catholic University of Sacred Heart, Faculty of Medicine, L.go F. Vito, 1-00168 Rome, Italy. Tel.: +39 06 3016619; fax: +39 06 3012753.
E-mail address: fwolf@rm.unicatt.it (F.I. Wolf).

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inconsistent results [6,7]. In vitro studies have explored the potential effects of ELF-EMF on cell proliferation [8], apoptosis [9,10], differentiation [11], genotoxicity [12] and proto-oncogene modulation [13–15]; again, the results remained inconclusive or open to debate. It is unclear how ELF-EMF would influence cellular behavior, but a plausible hypothesis is that ELF-EMF affect membrane structure and permeability to small molecules. In a recent study, we have shown that in neuroendocrine cells ELF-EMF exposure increased Ca^{2+} currents due to overexpression of voltage-gated Ca^{2+} channels, an occurrence that may well be correlated with proliferative events [16]. Another interesting hypothesis is that ELF-EMF interfere with chemical reactions involving free radical production (see Ref. [17] for review).

It is well established that free radicals have pleiotropic effects which may vary from cytotoxic to mitogenic responses depending on the dose intensity, the duration of exposure, and the type of cell or tissue [18]. Free radicals, e.g., hydroxyl radicals, can interact with DNA and form primarily 8-OHdG adducts, resulting in single strand breaks [19]. Adducts or strand breaks are recognised and usually removed quite efficiently by specific repair mechanisms. Nevertheless, DNA damage could become a site of mutation and a key step to carcinogenesis if the damage were extensive enough to overcome the repair capacity of the cell [20,21]. On the other hand, it has been demonstrated that low levels of reactive oxygen species trigger intracellular signals that involve the transcription of genes and lead to responses including proliferation [22,23]. One of the best characterized redox-modulatable signals involves NFκB, which can trigger proliferation or apoptosis [24,25]. NFκB/Rel proteins, primarily composed of heterodimers of p50/p65, are present in the cytoplasm in their inactive form associated with IkBs. Activation occurs after phosphorylation of IκBα and translocation of p50/p65 into the nucleus where, after further phosphorylation of the p65 subunit, it binds to DNA and functions as a transcription factor. In several studies, overexpression and nuclear translocation of p65 were shown to correlate with the transcription activity of NFkB [26]. Activation of NFκB through phosphorylation of IκB has also been investigated in detail and shown to be associated with receptor-mediated signals like CTLA4 [27]. Other activation mechanisms, mediated by redox reactions, have also been described.

In this study we evaluated the effects of 50-Hz ELF-EMF on cell proliferation, cell cycle distribution and DNA damage in normal cells (embryonic human lung fibroblasts, WI-38), neoplastic cells (human promyelocytic leukemia cells, HL-60), and immortalized cells (rat fibroblasts, Rat-1). We show that 24–72-h exposure to 0.5–1-mT ELF-EMF increased cell proliferation and DNA damage in all cell types, and that these effects might be mediated by formation of reactive oxygen species.

2. Materials and methods

2.1. Cell culture

Human promyelocytic HL-60 cells were grown at 37 °C in 5% CO_2/air atmosphere in RPMI 1640 (Sigma Italia, Milano) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies Italia, Milano). Rat-1 fibroblasts were cultured at 37 °C in 5% CO_2/air atmosphere in EMEM medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Life Technologies). WI-38 diploid fibroblasts derived from embryonic human lung primary culture were purchased from Istituto Zooprofilattico dell'Emilia-Romagna (Brescia, Italy). Cells were received from the provider at 11 population doublings (PDs) and were grown at 37 °C under 5% CO_2/air atmosphere in Eagle basal medium (BME) (Sigma Italia) supplemented with 10% fetal bovine serum (FBS) (Life Technologies Italia) and 1 mM sodium pyruvate. Cells were used for experiments within 21 PDs.

Experiments were routinely carried out on triplicate cultures. At specified times cells were harvested and duplicate hemocytometer counts with Coulter Z1 were performed. The trypan blue exclusion test was used to evaluate the percentage of viable cells. In all cases, cells were used for experiments only when viability was >95%.

2.2. Anti-oxidant treatment

Where indicated, cells were exposed to the antioxidant DL-α-tocopherol (Fluka Chemika-bioChemika, Buchs, Switzerland). After 24 h the medium was replaced and cells were exposed to ELF-EMF. DL-α-Tocopherol was delivered to the cells using tetrahydrofuran (THF) or 96% ethanol (Fluka) as a solvent. When THF was used 0.25 g/L BHT was added to avoid the formation of peroxides. Stock solutions of α-tocopherol were prepared immediately before each experiment and judged ~98% pure by HPLC. Aliquots of stock solutions were rapidly added to the culture medium to achieve a final concentration of 10 μM. Control experiments showed that 10 μM was the lowest concentration at which α-tocopherol interfered with ELF-EMF while not eliciting any direct effect on cell proliferation (data not shown). The amount of THF added to the cells never exceeded >0.5% (v/v). Control experiments showed no difference between untreated cultures and those treated with THF or ethanol alone in terms of cell number, viability, and oxidative events (ROS production and 8-OHdG formation). Where indicated, "control cells" therefore refers to untreated cells.

2.3. Electromagnetic field exposure

A solenoid field generator producing sinusoidal waveform with amplitude of 0.05–1.0 mT and frequency of 1–100 Hz was placed in a tissue culture incubator. For technical details, see Ref. [16]. Control and exposed cells were placed in the same incubator outside or inside the

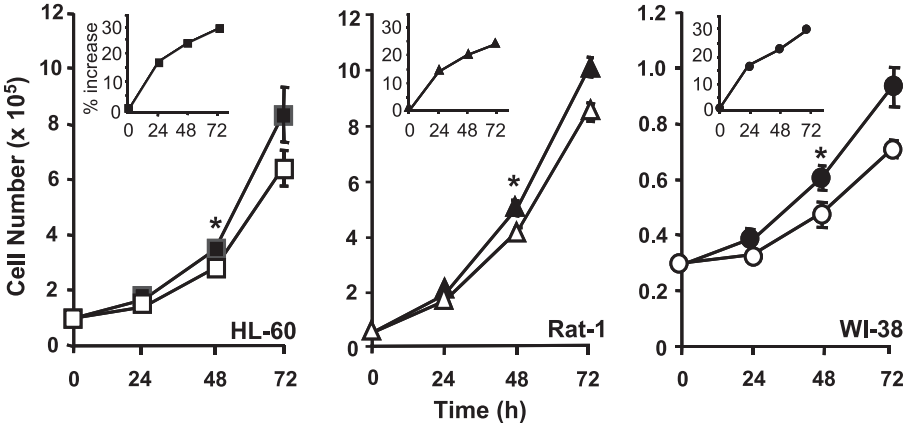

Fig. 1. Effect of ELF-EMF exposure to 1.0 mT/50 Hz for 72 h on proliferation of HL-60 cells, Rat-1 and WI-38 fibroblasts. After plating cells were grown for 72 h under continuous ELF-EMF exposure (closed symbols) or in control condition (open symbols). Every 24 h cells were evaluated by an automatic Coulter counter. Inserts report % net growth increase of exposed compared to control cells. Data are mean±S.D. of five separate experiments; *P<0.05 vs. control cells by Student's t test.

solenoid, respectively. Cells were exposed to a sinusoidal 50-Hz electromagnetic field at 0.50, 0.75 and 1.0 mT for 3–72 h in culture dishes inserted into plexiglas cylinder placed inside the solenoid. A thermometric probe placed in cell culture dishes inside and outside the ELF-EMF generator revealed no significant temperature difference between culture media of exposed or unexposed cells. After treatment, cells were washed and rapidly used for the assays.

2.4. Assays for 8-OHdG and DNA strand breaks

Cytospin samples were prepared according to the following procedure. Cells were diluted in sucrose buffer (0.25 M sucrose, 1.8 mM $CaCl_2$, 25 mM KCl, 50 mM Tris, pH 7.5) at a density of about 2×10^6 cells/ml. Next, 50 μl was added to carbowax-ethanol buffer (carbowax stock: 77-ml PEG-1000 in 50-ml water, 1-ml stock in 74-ml 70% ethanol) (Sigma Italia) and mixed. Aliquots of 150 μl were placed into cytospin funnels and centrifuged at 300 rpm for 5 min. Slides were coated with aminopropyl-triethoxysilane (Kindler, Freiburg, Germany). Samples were air-dried for 10–30 min, fixed in 95% cold ethanol (−20 °C) for 10 min, and stored at −20 °C.

Detection of 8-OHdG by immunocytochemistry coupled with DAB (Vector, Burlingham, USA) was carried out essentially as described by Yabourough et al. [28]. The monoclonal antibody for 8-OHdG 1F7 was kindly provided by Dr. R.M. Santella, Columbia School of Public Health, NY. Semi-quantitative evaluation of the staining was carried out by an optical microscope (ECLIPSE E600, Nikon, at 400×) connected to an Image-Pro plus Version 4.1 (Media Cybernetics, USA). Nuclear staining was evaluated in approximately 100 cells of randomly chosen images by operators who were blind to the status of cell treatment, as recommended in Ref. [28]. Negative and positive controls (untreated and 0.5 mM H_2O_2-treated cells, respectively) were included within each batch of slides. Data are reported as units of optical density (OD) ×1000.

Detection of SSB by single cell microgel electrophoresis was performed by the method of Singh [29], with minor modifications [30]. Data are reported as tail moment [31], evaluated by Image-Pro plus Version 4.1. At least 50 randomly selected representative comets were calculated for each blind sample.

2.5. Cytofluorimetric analysis of cell cycle distribution

Trypsinized cells were collected and washed twice with PBS. About 1×10^6 cells were suspended in 1-ml PBS, fixed in 5 ml of 70% ethanol and stored at 4 °C. At the time of analysis, cells were collected by centrifugation and the pellets were resuspended in 0.2 mg/ml propidium iodide in PBS containing 0.6% Nonidet P-40 and RNAase (1 mg/ml); suspensions were incubated in the dark at room temperature for 30 min. The cell suspensions were then filtered and analyzed for DNA content on Coulter EPICS 753 flow

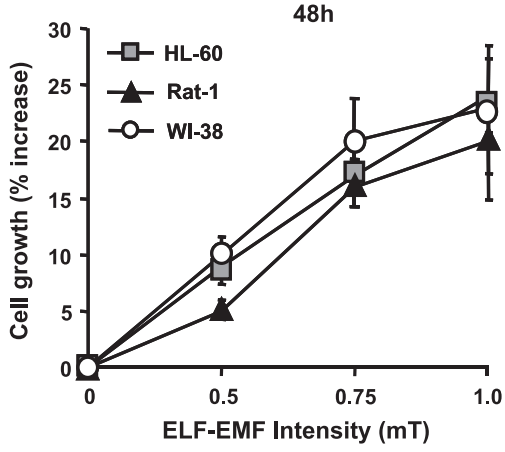

Fig. 2. Effect of ELF-EMF exposure from 0.5 to 1.0 mT/50 Hz for 48 h on proliferation of HL-60 cells, Rat-1 and WI-38 fibroblasts. Cells were exposed to ELF-EMF of 0.5, 0.75, and 1.0 mT/50 Hz for 48 h and their growth rate compared to matched control cultures. Data (mean±S.D. of five experiments) are expressed as % growth increase.

Table 1
Effect of ELF-EMF (1 mT–50 Hz) on cell cycle distribution in Rat-1 cells

	Cell cycle distribution (%)							
	12 h		24 h		48 h		72 h	
	G0/G1	S	G0/G1	S	G0/G1	S	G0/G1	S
Control	50.6	31.5	67.7	20.1	55.4	27.5	57.4	24.0
ELF-EMF	26.3	38.8* (+23%)	63.5	23.5 (+17%)	52.2	34.3* (+25%)	67.3	17.0 (−30%)

Data are means of three experiments, S.D. being <15%.
* $P<0.05$ by Student's t test.

cytometer. The percentage of cells in the different phases of the cell cycle was determined using the Multicycle software version 2.53.

2.6. Western blot analyses of protein expression

Cells (10×10^6) were harvested and pellets were suspended in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 0.05% NaN_3, 1% Triton and 1 mM PMSF) for 30 min at 4 °C. After incubation samples were centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatants were assayed for protein content by the Biorad protein assay method (Biorad laboratories GmbH, Munchen, Germany) and stored at −80 °C. Western blot analysis was performed with equal amounts of proteins from each sample (generally <100 μg), separated by SDS-PAGE (12%) and transferred to immobilon-P membranes (Millipore, Bedford, MA) at 100 V for 1 h. Immunodetection was performed using the enhanced chemiluminescence kit for western blotting detection (Amersham Pharmacia Biotech, Freiburg, Germany). The polyclonal antibodies to p65, p50 and IκBα (1:1000 dilution) were from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal antibody to β-actin (1:200) (Santa Cruz Biotechnology) was used as internal control.

2.7. Intracellular ROS evaluation

Intracellular ROS were detected in dichlorodihydrofluorescein-diacetate (H_2DCF-DA)-loaded cells (Molecular Probe, Leiden, Netherlands), using a Cytofluor 2300/2350 (Millipore, Billerica, MA). Samples of 2×10^6 cells placed on Corning 6-wells plates were preincubated with 5 μM H_2DCF-DA for 1 h at 37 °C. Plates were centrifuged at 1200 rpm for 10 min and the fluorescence of control and treated cells was read in the Cytofluor (excitation at 504 nm, emission at 526 nm). Alpha-tocopherol at the concentration of 10 μM did not alter the basal fluorescence of DCF (Background of DCF was usually 60–70 relative fluorescent units/F.U.).

2.8. Statistical analysis

Data are given as means±S.D. of at least three separate experiments. In the figures, S.D. are indicated by vertical bars; values without vertical bars have S.D. within the symbols. Statistical analyses were performed by unpaired Student's t test, and differences were considered significant when $P<0.05$.

Multifactorial two-way analysis of variance (ANOVA) was adopted to assess differences among multiple sets of data obtained with untreated or treated cells at different times of culture and exposure. When significant values were found ($P<0.05$), post hoc comparisons of means were made using the Tukey's Honestly Significant Differences test. Other details are given in the legends to figures and tables.

3. Results

3.1. Effect of ELF-EMF on proliferation

We investigated the effects of 50 Hz ELF-EMF exposures of different intensities (from 0.5 to 1.0 mT) for up to 72 h on

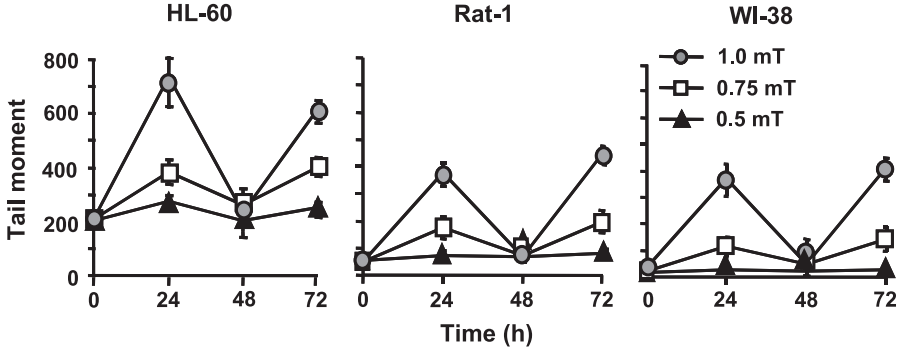

Fig. 3. Effect of ELF-EMF exposure at 0.5–1.0 mT/50 Hz for 72 h on DNA damage evaluated as strand breaks in HL-60 cells, Rat-1 and WI-38 fibroblasts. Cells were exposed for different extent of time to ELF-EMF from 0.5 to 1.0 mT/50 Hz and single strand breaks were evaluated by Comet assay. Data (mean±S.D. of three experiments) are expressed as tail moment. Values of basal damage were subtracted from all points. See Materials and methods for technical details.

Table 2
Effect of α-tocopherol pretreatment of ELF-EMF-induced proliferation

	Increase of cell growth (%)					
	HL-60		Rat-1		WI-38	
Exposure (h)	ELF-EMF	+α-T[a]	ELF-EMF	+α-T	ELF-EMF	+α-T
24	17.0	1.4 (−15.6)**	14.0	6.5 (−7.5)*	17.2	2.0 (−15.2)**
48	24.0	24.0	20.0	12.2 (−7.8)*	23.0	8.7 (−14.3)**
72	29.1	29.0	24.3	24.0	31.0	2.2 (−28.8)**

[a] Cells were preincubated with 10 μM α-tocopherol for 24 h prior to exposure to ELF-EMF. Note that 10 μM α-tocopherol did not influence proliferation rate in non-exposed control cells during 24–72-h incubations.
* $P<0.05$ by Student's t test.
** $P<0.005$ by Student's t test.

the proliferation rate of HL-60 leukemia cells, Rat-1 immortalized fibroblasts, and WI-38 diploid fibroblasts. Fig. 1 shows that 1-mT ELF-EMF increased the proliferation of the three cell types in a time-dependent manner, reaching a statistical significance vs. unexposed cells after 48-h exposure ($P<0.05$). The increases in proliferation rate were proportional to the exposure time, as shown in the insets in which data were expressed as percent relative to unexposed cells. At 72 h, the growth rate increased 20–30% in all cells. Fig. 2 shows that in all cell types ELF-EMF enhanced cell proliferation in a dose-dependent manner. After 48-h exposure to 0.75 mT the average cell growth increased by approximately 15–20%. Since the effect of ELF-EMF was proportional to the dose intensity, all subsequent experiments were performed at 1.0 mT, so as to maximize the effects induced by ELF-EMF.

To confirm data obtained by cell counts, we measured time-related changes in cell cycle distribution during the course of 72-h exposure to ELF-EMF (1 mT/50 Hz). Table 1 reports data obtained with Rat-1 cells. Compared to controls, ELF-EMF-exposure caused a significant increase of the percentage of cells in S phase at 12 h and at 48 h. At 72 h the cells in S phase decreased by 30% suggesting that, under these conditions, the exposed cells reached confluence earlier than controls. Similar results were obtained with HL-60 cells and WI-38 fibroblasts (data not shown).

3.2. Effect of ELF-EMF on DNA damage

Fig. 3 reports DNA damage, measured as DNA strand breaks by the comet assay, in the three cell lines after 72-h exposure to 0.5–1.0-mT ELF-EMF. At time 0, the basal levels of strand breaks were substantially higher in HL-60 cells than in Rat-1 and WI-38 fibroblasts, as one would expect to find in a hyperdiploid neoplastic population [32]. Irrespective of the basal levels of DNA damage, however, 24-h exposure to ELF-EMF increased strand breaks in all cell lines in a dose-dependent manner. This effect was much evident at 1.0-mT ELF-EMF, especially in non-neoplastic cells like Rat-1 (eightfold increase) and WI-38 fibroblasts (16-fold increase). In the following 24 h of exposure, strand breaks returned to basal levels but increased again at 72 h. It is worth noting that DNA damage appeared 12 h after the peak of S phase (cf. Table 1). The repair of DNA damage after 72-h exposure to ELF-EMF was also investigated. After 24 h of post-exposure recovery Rat-1 cells showed levels of DNA damage that were reduced by about 92% compared to the levels determined at the end of 72-h exposure to ELF-EMF (net tail moments, 30 vs. 399). Under comparable conditions HL-60 cells showed levels of DNA strand breaks that decreased only 44% (net tail moments, 234 vs. 414). This latter finding did not come unexpected, as we

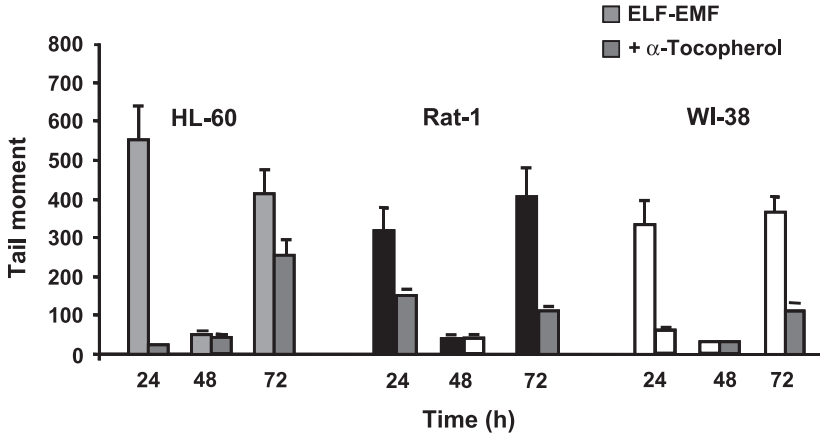

Fig. 4. Effect of antioxidant treatment on DNA damage induced by ELF-EMF at 1.0 mT/50 Hz up to 72 h in HL-60 cells, Rat-1 and WI-38 fibroblasts. Cells were pretreated with 10 μM α-tocopherol for 24 h. After removal of excess antioxidant, treated cells were exposed to ELF-EMF from 24 to 72 h. Data expressed as tail moment are mean±S.D. of three different experiments. Values of basal damage were subtracted from all points.

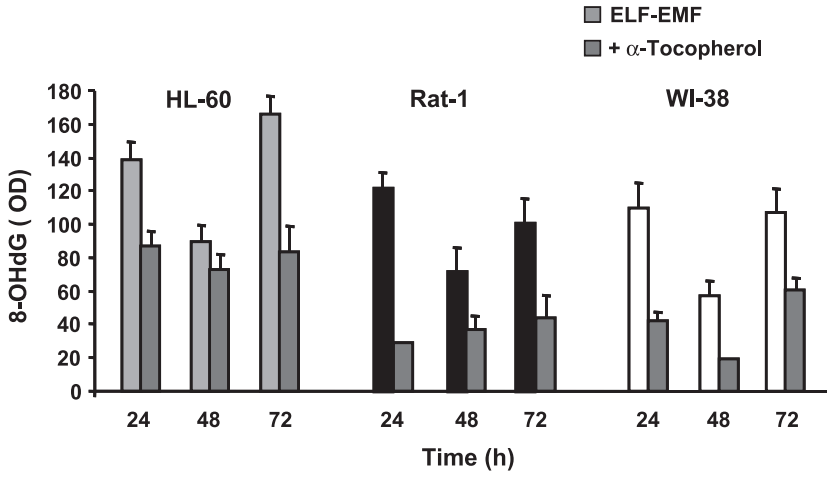

Fig. 5. Effect of antioxidant treatment on oxidative DNA damage evaluated as 8-OHdG, induced by ELF-EMF at 1.0 mT/50 Hz up to 72 h in HL-60 cells, Rat-1 and WI-38 fibroblasts. Cells were pretreated with 10 μM α-tocopherol for 24 h prior to exposure to ELF-EMF from 24 to 72 h. 8-OHdG adducts identified by the monoclonal antibody 1F7 coupled with DAB were quantified evaluating optical density (OD) (see Materials and methods for further details). Basal staining was subtracted from experimental data. Data are means±S.D. of three different experiments.

have previously shown that neoplastic cells are characterized by insufficient repair mechanisms [32].

3.3. Effect of antioxidant on ELF-EMF proliferation and DNA damage

Cells were treated with an antioxidant prior to their exposure to ELF-EMF. α-Tocopherol at 10 μM, added as described under Materials and Methods [33], prevented stimulation of cell proliferation in all cell populations examined after 24-h exposure to ELF-EMF (Table 2). After 48-h exposure the same effect was present in Rat-1 and WI-38 cells, but not in HL-60 cells; after 72-h exposure, α-tocopherol inhibited proliferation only in WI-38 cells (see also Table 2). Similar results were obtained when the effects of α-tocopherol on DNA damage were evaluated. Fig. 4 shows that α-tocopherol significantly reduced DNA strand breaks in all cell strains, and this effect was more persistent in Rat-1 and WI-38 cells than in HL60.

To better characterize whether DNA was damaged by oxygen-centered free radicals, we measured the levels of 8-OHdG adducts, which indicate oxidative damage by hydroxyl radicals or hydroxyl radical-type species [20]. Fig. 5 shows that in all cell types 8-OHdG levels peaked at 24 and 72 h after exposure to ELF-EMF, similar to that determined by the comet assay (see Fig. 4). Alpha-tocopherol prevented 8-OHdG formation by ~50% throughout ELF-EMF treatment in all cell types.

3.4. Involvement of ROS in ELF-EMF induced DNA damage and proliferation

Having shown that the effects of ELF-EMF on cell proliferation and DNA damage were inhibited by antioxidants, we performed experiments to obtain direct evidence that ELF-EMF caused the formation of free radical species. We therefore measured intracellular ROS in DCF-loaded Rat-1 fibroblasts. Fig. 6 (panel A) shows that the cellular levels of ROS increased ~18% as early as 3 h after exposure to ELF-EMF and that such an increase persisted after 24-h exposure. Under comparable conditions, α-tocopherol

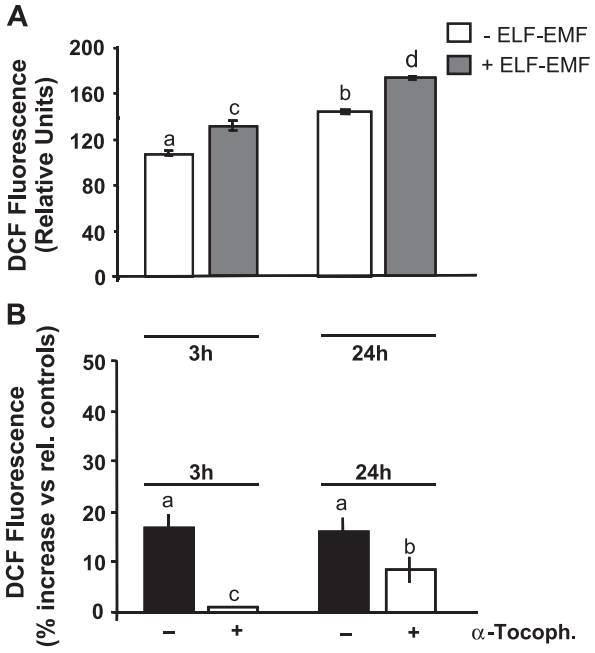

Fig. 6. Effects of ELF-EMF exposure at 1.0 mT/50 Hz on ROS production in Rat-1 fibroblasts with or without α-tocopherol pretreatment. DCF-detectable ROS were measured in control or ELF-EMF-exposed cells (3- and 24-h exposures). Values were expressed as relative fluorescence units. Panel A shows that treatment/time interaction was significant ($P<0.05$). Values not sharing the same superscript were significantly different (c and d: $P<0.05$ vs. a and b, respectively). Panel B shows the effect of 24-h pretreatment with 10 μM α-tocopherol on ELF-EMF-induced ROS production. Data are reported as percent of DCF fluorescence increase vs. relative controls at 3 and 24 h of exposure to ELF-EMF (b: $P<0.05$ vs. a; c: $P<0.001$vs. a) (Tukey's test).

almost completely prevented ROS increase at 3-h exposure, and reduced ROS increase by ~50% at 24-h exposure (Fig. 6, panel B).

3.5. Effect of ELF-EMF on redox-mediated signals and proliferation

We determined possible changes in the expression levels of proteins that are involved in redox-mediated signals. Fig. 7A shows the expression of NFκB p65 and p50 in Rat-1 cells. It can be observed that both NFκB p65 and p50 expression was increased at 12 h of exposure to 1 mT/50 Hz ELF-EMF. Compared to controls, p65 expression increased 120% at 12 h and remained high up to 24 h. The increase of p50 expression was relatively smaller but still evident and persistent at both 12 and 24 h. Under comparable conditions, pretreatment with 10 μM α-tocopherol did not modify p65 expression in control and exposed cells (Fig. 7B). The effects of ELF-EMF on the levels of the total inhibitory subunit IκBα were also investigated. Whereas in control cells total IκBα tended to increase from 6 to 24 h, in cells exposed to ELF-EMF IκBα underwent a significant decrease; of note, pretreatment of cells with α-tocopherol increased total IκBα both in the absence and presence of ELF-EMF (Fig. 8A). To better evaluate the functional implications of the modulation of total IκBα, we determined the p65/IκBα ratio as an index of NFkB activity. Fig. 8B shows that (i) cells exposed to ELF-EMF displayed a significant increase of the active form of NFκB, which peaked at 6 h and remained higher than in control cells at 12 and 24 h exposure; (ii) pretreatment of cells with α-tocopherol always abolished the increased ratio of p65 to IκBα induced by ELF-EMF. These data confirmed that redox-mediated signals were involved in the control of cell proliferation induced by ELF-EMF.

4. Discussion

In this study we utilized human lymphoblastic leukemia (HL-60 cells), immortalized but not transformed fibroblasts (Rat-1), and human diploid fibroblasts (WI-38 cells) with the aim of investigating the effect of ELF-EMF on normal and tumor cells. Our results showed that ELF-EMF dose-dependently increase cell proliferation, leading to a 30% increase of cell number after 72 h exposure (cf. Figs 1 and 2). Increased proliferation was paralleled by a significant

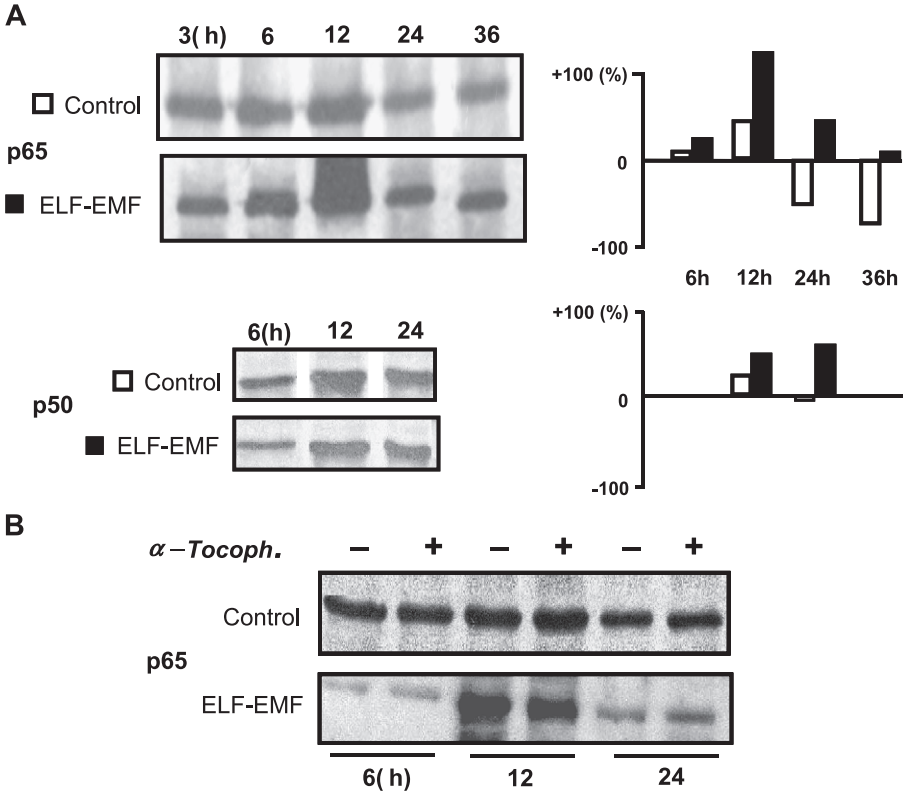

Fig. 7. Expression levels of p65 and p50 NFkB-related proteins in Rat-1 cells exposed to ELF-EMF 1 mT/50 Hz for different extent of time. Effect of antioxidant pretreatment. Proteins from control and ELF-EMF-exposed cells from 3 to 36 h were run on a gel electrophoresis and stained with monoclonal antibodies for p65-Rel1, p50 and α-actin as housekeeping protein. Panel A shows the expression of p65 and p50 from 3- to 36-h incubation in control and exposed cells (70-μg protein was loaded into the gel). On the left, densitometric evaluations show the percentage increase of protein expression compared to values at 3 h for p65 and at 6 h for p50, respectively. Panel B reports the effect of 24-h pretreatment with 10 μM α-tocopherol on the expression level of p65 from 6- to 24-h incubations (40-μg protein loaded into the gel). Results confirmed by two separate experiments.

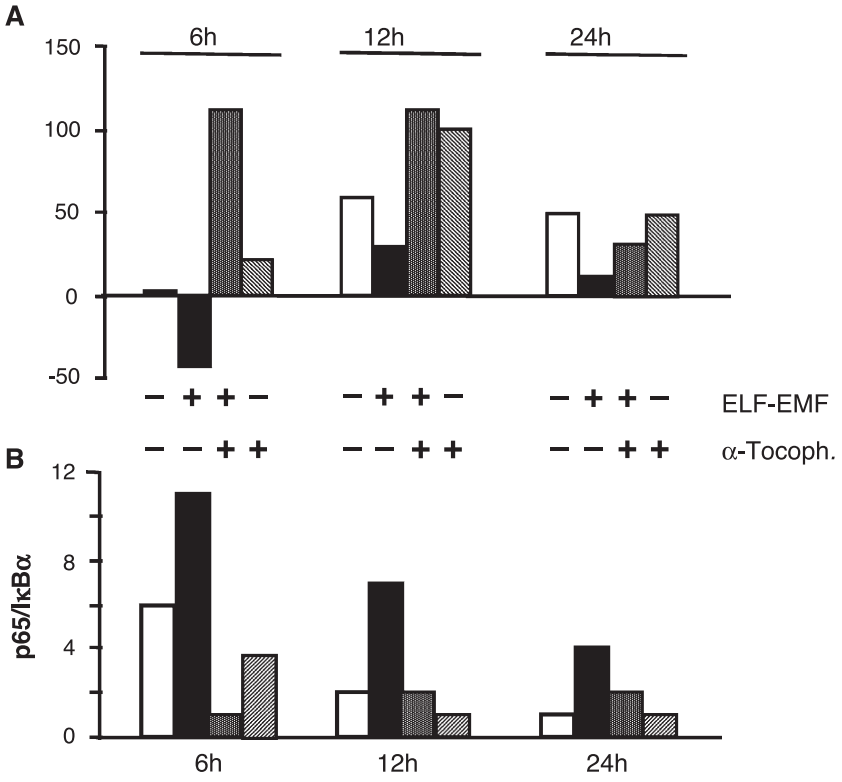

Fig. 8. Expression levels of IκBα, the NFkB-inhibitory subunit, in Rat-1 cells exposed to ELF-EMF 1 mT/50 Hz with or without antioxidant. Panel A: The expression levels of IκBα, normalized to α-actin densitometric quantification, are expressed as percentage of the control cells at 6-h culture. When indicated, cells were pretreated with 10 μM α-tocopherol for 24 h prior to incubation with or without ELF-EMF exposure. Panel B: Ratio of the expression levels of p65 to IκBα (p65/IκBα), an index of NFkB active form. Results confirmed by two separate experiments.

increase of the percentage of cells in the S phase, which reached a maximum of 25% in the case of Rat-1 cells after 12-h exposure. Interestingly, the enhanced recruitment of cells in the S phase displayed two peaks after 12- and 48-h exposure. Parallel evaluation of DNA damage showed that ELF-EMF significantly increased both DNA strand breaks and 8-OHdG levels, which reached their maxima after the S phase peaks (cf. Figs 3 and 4 and Table 1). This observation suggests that the exposure to ELF-EMF caused a transient mitogenic effect at 12 h, followed by a DNA damaging effect at 24 h.

From a pathogenetic view point, it can be hypothesized that short-term exposures to ELF-EMF, and consequent formation of ROS, induce growth stimulation and increase the number of DNA synthesizing cells. Long-term exposures to ELF-EMF and continuous generation of ROS eventually cause accumulation of DNA damage, which slows down the progression of the cell cycle. Collectively, the percentage of cells in the S phase was inversely related to DNA damage and directly related to DNA repair (cf. Table 1 and Fig. 3). These results are consistent with the recent evidence for a relationship between cell cycle distribution and susceptibility to oxidative DNA damage [34]. According to this interpretation cell cycle arrest in G1 protects against oxidative DNA damage, demonstrating an inverse relationship between cell proliferation and oxidative DNA damage.

Reactive oxygen species have been tentatively proposed to mediate the effects of ELF-EMF [35]. We have extended and validated this concept by demonstrating that a sizable increase of ROS occurred shortly after exposure to ELF-EMF and preceded activation of molecular events able to induce cell proliferation (cf. Figs. 6–8). In addition, we have shown that an antioxidant like α-tocopherol blunted the increase of ROS and consistently prevented proliferation and DNA damage induced by ELF-EMF (cf. Table 2 and Figs. 4–7). It is worth noting that the effect of α-tocopherol was more persistent in WI-38 cells than in HL-60 cells, and exhibited an intermediate duration in Rat-1 cells (cf. Table 2 and Fig. 4). This might be attributed to cell-specific rates of incorporation and metabolism of α-tocopherol, as shown in previous studies of other antioxidants [36].

We also provide novel information about the mechanism whereby ROS can mediate the effects of ELF-EMF on cell proliferation. In fact, our data demonstrate that ELF-EMF increase the levels of NFκB-related proteins, most notably p65, while also decreasing the levels of IκBα (cf. Figs 7 and 8). The increased levels of p65, in conjunction with the reduced levels of total IκBα, have a functional role in regulating transcription processes, as p65-p65 [37] homodimers or p50-p65 heterodimers [26] are known to activate transcription. Antioxidant pretreatment with α-tocopherol does not interfere with the effects of ELF-EMF on p65 or p50 but increases the levels of IκBα in such a manner that

the ratio of p65 to IκBα returns to essentially the same levels of untreated cells. By doing so, α-tocopherol precludes nuclear translocation of p65-p50 active complexes [38].

The precise mechanisms through which ELF-EMF increase cellular ROS production remain unknown. Likewise, we cannot speculate which particular reactive species mediated the proliferative and DNA-damaging effects and how such reactive species could be scavenged by α-tocopherol. Dichlorofluorescein is widely used for detecting cellular ROS, and reportedly exhibits a peculiar specificity for hydrogen peroxide [39]; however, chemical studies show that DCF actually detects a much broader array of reactive intermediates [40]. Alpha-tocopherol is one of the most effective lipid-soluble antioxidants and, as such, would be expected to scavenge lipid centered radicals rather than hydrogen peroxide or the hydroxyl radicals that eventually form 8-OHdG adducts such as those detected in our study. Nonetheless, previous studies showed that α-tocopherol inhibited the formation of DCF-detectable ROS in cells [41], and was able to diminish DNA strand breaks induced by hydrogen peroxide [42]. Our findings that α-tocopherol decreases the yield of DCF-detectable ROS in cells exposed to ELF-EMF, while also diminishing proliferation and DNA oxidative damage, must therefore be appreciated within the context of an oxidizing tone in which more than one oxidant was involved and α-tocopherol probably acted on more than one free radical species.

The biological effects of ELF-EMF remain a matter of debate, as indicated by recent papers supporting [8,43–46] or disproving [47–49] an effect of ELF-EMF on cell growth and DNA damage. These discrepancies might at least in part be attributed to experimental factors like intensity and duration of exposure or the cell types used in the different studies. Here we have shown that ELF-EMF first increase proliferation and then induce reversible DNA damage in normal and leukemic cell cultures characterized by different growth rates. Thus, our model shows that both cell growth and DNA damage can be observed in cell populations that are exposed to ELF-EMF for a sufficiently long time and then are allowed to recover after treatment. The consequences of these dual and time-dependent effects would depend on the DNA repair capacity of cells, or the accumulation of potentially carcinogenic mutations, or the coupling of DNA damage with apoptosis. Having one consequence or the other will also depend on environmental or nutritional factors that modulate the impact of ELF-EMF on the cell. These results may therefore be of value to develop research and new investigational models on the potentially deleterious effects of ELF-EMF.

In conclusion, the data reported in this paper confirm that ELF-EMF influence cell proliferation and susceptibility to DNA damage, and uncover mechanisms through which ROS may be important in these settings. Such effects are seen in normal or transformed cells with very different proliferation rates, and thus highlight the importance of characterizing the role of ELF-EMF in the complex process of carcinogenesis or tumor progression.

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