Field exposure to 50 Hz significantly affects wild-type and unfolded p53 expression in NB69 neuroblastoma cells

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Abstract. Previous studies have shown that intermittent exposure to a 50 Hz, 100 μT sinusoidal magnetic field (MF) promotes proliferation of human neuroblastoma cells, NB69. This effect is mediated by activation of the epidermal growth factor receptor through a free radical-dependent activation of the p38 pathway. The present study investigated the possibility that the oxidative stress-sensitive protein p53 is a potential target of the MF, and that field exposure can affect the protein expression. To that end, NB69 cells were exposed to short intervals of 30 to 120 min to the aforementioned MF parameters. Two specific anti-p53 antibodies that allow discrimination between the wild and unfolded forms of p53 were used to study the expression and cellular distribution of both isoforms of the protein. The expression of the antiapoptotic protein Bcl-2, whose regulation is mediated by p53, was also analyzed. The obtained results revealed that MF exposure induced increases in p53 gene expression and in protein expression of the wild-type form of p53. Field exposure also caused overexpression of the unfolded form of p53, together with changes in the nuclear/cytoplasmic distribution of both forms of the protein. The expression of protein Bcl-2 was also significantly increased in response to the MF. As a whole, these results indicated that the MF is capable of interacting with the function, distribution and conformation of protein p53. Such interactions could be involved in previously reported MF effects on NB69 proliferation promotion.

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Introduction

Several epidemiological studies have reported potential associations between chronic exposure to power frequency (50-60 Hz) magnetic fields (MFs) and increased risk of a number of pathologies, including amyotrophic lateral sclerosis, brain tumors or childhood and adult leukemia (1-8). Based primarily on epidemiological evidence on childhood leukemia, the International Agency for Research on Cancer (IARC) classified extremely low frequency [(ELF) MFs: 3 Hz-3 kHz)] as possible carcinogens to humans, class 2B (9). In addition, there is *in vitro* experimental evidence that provides partial support to the epidemiological data, as it reveals that exposure to ELF MFs can affect different cellular processes involved in cancer promotion (10,11). While it is accepted that ELF fields cannot directly damage the DNA molecule (12), exposure to a 50-Hz MF at flux densities as low as 0.1-1 mT has been reported to induce changes in DNA integrity (13-15) and it has been proposed that alterations in genes related to DNA repair, observed in acute leukemia patients, could be associated to chronic exposure of ELF MFs (16). This type of evidence has led to a number of hypotheses on potential mechanisms through which ELF fields could indirectly affect the DNA structure (17-20).

Tumor suppressor genes such as TP53 are involved in various processes associated with cell division, including gene expression regulation, cell cycle control, cell death programming or genome stability (21). Loss of activity of these genes can cause inability of processes controlling cell proliferation and occasionally lead to the development of neoplasms and to their evolution towards more aggressive tumor processes (22). Due to its central role in coordinating the cellular responses of a wide range of stressors, the TP53 gene has been described as a 'genome guardian' (23) or a 'cell guardian' (24). When DNA damage occurs, levels of the protein encoded by TP53 rapidly increase and the cell cycle stops at phase G1-S, allowing the cell a time span for DNA repair systems to act (25). Such a response does not occur in tumor cells whose TP53 gene is inactivated due to mutation, protein-protein interaction or reaggregation (26,27). In addition, inactivation of this tumor suppressor gene, which is a frequent event in tumorigenesis, has also been reported to be due to alterations

in the conformation of the wild-type (wt) p53 protein that do not necessarily involve mutations (28-30). Other factors potentially capable of compromising the functions of p53 are the inactivation of co-activators of the wt protein, the inactivation of downstream targets of p53 or the cytoplasmic retention of p53. Indeed, cytoplasmic sequestration of wt p53 has been observed in undifferentiated neuroblastoma, colon carcinoma and breast cancer cells (31,32).

In addition to exerting the aforementioned described transcription-dependent functions at the nuclear domain, p53 interacts with cytoplasmic proteins such as those of the Bcl-2 family, leading to permeabilization of the mitochondrial membrane and increased apoptosis (33-35). Moreover, in contrast to wt p53, endogenous missense mutants of p53 are unable to form complexes with endogenous Bcl-2 in human cancer cells, which renders them unable to induce apoptosis (36).

Recently, the mitogen-activated protein kinase (MAPK) has been reported to integrate extracellular signals related to p53 expression and its role in cell cycle regulation (37). In previous studies by the authors, it was demonstrated that intermittent exposure to a weak, 50-Hz MF, in addition to increasing free radical levels in NB69 human neuroblastoma cells, promoted the activation of the MAPK-ERK1/2 and -p38 transduction pathways, as well as that of the EGF receptor. Such activations, some of which are stress-dependent, induce cell cycle changes that lead to a significant increase in the proliferation of NB69 cells (38-40). Based on these data, the present study investigated whether *in vitro* exposure to a 50-Hz, 100-μT MF can affect the expression of protein p53 in NB69 cells. Two conformational specific anti-p53 antibodies were used to that end: pAb240, which specifically recognizes the unfolded p53 tertiary structure, and pAb1801, which recognizes the folded, wt isoform (41-43). The results revealed that MF exposure causes changes in both gene and protein expression of wt p53, as well as overexpression of unfolded p53, together with changes in its nuclear/cytoplasmic distribution. Additionally, MF exposure induced significant overexpression of the anti-apoptotic protein Bcl-2.

Materials and methods

Cell culture. The neuroblastoma cell line NB69 (lot no. 03I019/2008; cat. no. 99072802) was purchased from the European Collection of Authenticated Cell Cultures (ECACC). The cells were periodically tested for *Mycoplasma* contamination (PCR) and response to chemical and physical treatments. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; cat. no. BE12-614F; BioWhittaker; Lonza Group, Ltd.) supplemented with 10% heat-inactivated foetal bovine serum (FBS; product code 11573397), 2 mM L-glutamine (product code 11539876), 100 U/ml penicillin, 100 U/ml streptomycin and 0.25 μg/ml of amphotericin B (product code 11580486; all Gibco BRL; Invitrogen; Thermo Fisher Scientific, Inc.) and incubated in a 95% air-5% CO_2 humidified atmosphere (Forma Scientific incubators; Thermo Fisher Scientific, Inc.). In each experimental run $4.5x10^4$ cells ml^{-1} were seeded either directly on the bottom of 60-mm plastic Petri dishes (cat. no. 150288; Nunc, LabClinics, S.A.) or on glass coverslips placed inside the dishes, and cultured for 4 days in the described incubation conditions before MF- or sham-exposure.

Magnetic field exposure. The cultures were exposed to a 50-Hz, sine wave, vertically polarized MF, at a magnetic flux density (B_{AC}) of 100 μT root mean square (rms). The MF exposure set-up has been previously described (44). Briefly, current flow was supplied by a wave generator (Newtronic, Model 200MSTPC; TER Calibration Ltd.) that has a 3.53 mA DC offset (B_{DC} =15 μT rms). The generator was connected to two identical coil pairs, both set in Helmholtz configuration. The current in the coils was monitored using a multimeter (Hewlett Packard, model 974A; Hewlett Packard Company) and the induced MF was routinely verified with two magnetometers (EFA-3; Wandel and Goltermann GmbH & Co. Elektronische Meûtechnik; and EMDEX II; Enertech Consultants). One coil pair was placed inside each of the two magnetically shielded chambers (Co-netic® metal; Amuneal Manufacturing Corp.) located within two identical CO_2 incubators. The background MF inside the shielded chambers was B_{AC}, 0.04±0.03 μT rms; and B_{DC}, 0.05±0.04 μT rms. No increase of temperature at the sample location was observed using two Pt100 Thermocouple probes (Fluke, Model 52; Adler Instruments) when the coils were energized to produce the desired magnetic flux density of 100 μT rms. In each experimental run, Petri dishes (5 per experimental group) containing cell samples were stacked in the central region of the Helmholtz coil gap, which ensured uniformity of MF exposure. Only one set of coils was energized in each experimental run. The samples in the unenergized set were considered sham-exposed controls. Following a random sequence, both coil sets and incubators were alternatively used for MF- or sham-exposure. The protein expression of signaling markers was analyzed at various time intervals after the MF- or sham-exposure onset: 30, 60, 90 or 120 min.

Western blot analyses. Total protein extraction and immunoblotting were performed as previously described (45). Briefly, whole cell proteins were prepared by lysing the cells in hypotonic lysis buffer (45). Protein content was determined by BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal protein volumes (60 μg) were separated from each of the samples, using 10% SDS/PAGE gels, and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare; Cytiva) by semidry transfer. The membranes were then incubated at room temperature with mouse monoclonal antibodies against proteins p53 (1:1,000; product no. 2524; Cell Signaling Technology, Inc.) or Bcl-2 (1:1,000; product no. MBS3017024; MyBiosource, Inc.). Anti-human β-actin (1:5,000; product no. A-5441, Sigma-Aldrich; Merck KGaA) was used as a loading control. Following washing, the membranes were incubated with anti-mouse IgG, horseradish peroxidase-linked whole antibody (product no. NA931; GE Heathcare; Cytiva) or with the fluorescent-labeled secondary antibody, IRDye 800CW, goat anti-mouse IgG (1:15,000; product no. 926-32350; LI-COR Biosciences), for 1 h at room temperature. For chemiluminescence detection and visualization of the immunoreactive bands, the enhanced detection kit ECL (RPN2132; GE Healthcare; Cytiva) and the ProXima image densitometer (Isogen Life Science B.V.) were used. The Odyssey infrared imaging system LI-COR was used to detect the signal from bands marked with

the fluorescent secondary antibody. The blots were analyzed by densitometric assay using PDI Quantity One-4.5.2 software (Bio-Rad Laboratories, Inc.). At least four experimental replicates were conducted for each of the studied proteins. Three MF-exposed dishes vs. three sham-exposed dishes per experimental run and per exposure period were used.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for p53 mRNA expression. Total RNA from NB69 cells was extracted with TRIzol reagent (product no. T9424; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol, at 90 min of MF- or sham-exposure onset. The RNA quality and quantity were assessed using NanoDrop ND-1000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and agarose gel electrophoresis (1%). A total amount of 500 ng RNA was reverse-transcribed using a Primer Script RT™ Reagent Kit (cat. no. RR037A; Takara Bio, Inc.). LightCycler 480 SYBR-Green I Master mix (product no. 04887352001) and LightCycler thermal cycler 480 II (both from Roche Applied Science) were used for real-time PCR. All determinations were triple analyzed, and the thermocycler protocol consisted of 5 min preincubation at 95˚C, followed by 45 cycles at 95˚C for 10 sec, 60˚C for 15 sec, and 72˚C for 10 sec. The following primers were used: TP53 forward, 5'-CAGCACATGACG GAGGTTGT-3' and reverse, 5'-TCATCCAAATACTCCACA CGC-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 1.1, 5'-GAAGGTGAAGGTCGGAGT C-3', and reverse, 5'-GAAGATGGTGATGGGATTTC-3'. The melting curves were evaluated and the PCR reaction products were separated on a 2% agarose gel to confirm the presence of a single product. In all cases, the efficiency of the amplification reaction was tested by serial dilutions of cDNA, ensuring that the efficiency was linear and close to 2. The results were analyzed using the relative quantification method described by Pfaffl (46), which takes into account the efficiency of the reaction for the target gene and the invariable control. GAPDH was used as an invariant endogenous control. For comparison and statistical analysis, data obtained were normalized to the expression of the control group.

Immunofluorescence. After MF- or sham-exposure of the samples cultured on coverslips, the expression of p53 and Bcl-2 were characterized by indirect immunofluorescence and computer-assisted image analysis. Cells were fixed with 4% formaldehyde for 20 min at 4˚C, permeabilized with ethanol/acetic acid for 20 min at -4˚C and blocked with PBS containing 10% goat serum (cat no. 31872; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Coverslips were incubated overnight at 4˚C with two different p53 primary mouse monoclonal antibodies: p53-pAb1801 (1:100; cat. no. AHO0122, Invitrogen; Thermo Fisher Scientific, Inc.) or p53-pAb240 (1:100; cat. no. 13-4100; Zymed Laboratories, Inc.; Thermo Fisher Scientific, Inc.). The conformational changes in p53 can be assessed by monitoring the reactivity with conformation-specific antibodies pAb1801 and pAb240, which allow discrimination between the functional, wt protein and its unfolded isoform (47,48). Antibody p53-pAb1801 recognizes an epitope between amino acids 32 and 79 of both wt and mutant-like type p53 protein (49). The conformationally altered mutant-like type isoform is specifically recognized by antibody pAb240 (43,50). This antibody recognizes a primary epitope that is cryptic in the wt conformation and becomes exposed when the protein changes its conformation towards an unfolded phenotype (51). Secondary anti-mouse-IgG conjugated to AlexaFluor® 568 (cat. no. A11031; Molecular Probes; Thermo Fisher Scientific, Inc.) was administered for 1 h at room temperature to reveal p53 expression. The cell nuclei were counterstained with Hoechst 33342 (product no. B2261; Bisbenzimide; Sigma-Aldrich; Merck KGaA) added to the mounting medium. The mouse monoclonal Bcl-2 primary antibody (1:100; product no. MBS3017024; MyBiosource, Inc.) and a biotinylated secondary antibody (1:100) were administered overnight at 4˚C and 1 h at room temperature, respectively. Immunostaining was enhanced through the ABC method (cat. no. PK-6102; Vectastain ABC kit; Vector Laboratories, Ltd.), and revealed with 3'3'-diaminobenzidine (DAB; product no. D5905; Sigma-Aldrich; Merck KGaA). The cell nuclei were counterstained with methyl-green for 5 min at room temperature. Images were captured with a Nikon microscope (Eclipse TE300; Nikon Corporation) and analyzed by computer imaging AnalySIS software (version 2007; Olympus Soft-Imaging Systems GmbH). In each of at least 4 experimental runs, 4 coverslips were studied per experimental condition: MF- or sham-exposed controls. A total of 15 microscopic fields per coverslip were randomly selected for analysis. The total number of nuclei and the percent of wt p53-, unfolded p53- or Bcl-2-positive cells per microscopic field were recorded. The cytoplasmic or nuclear location of protein p53 was determined by computer-assisted image-analysis (AnalySIS software, version 2007; Olympus Soft-Imaging Systems GmbH).

Statistical analysis. All experimental procedures and analyses were conducted blindly for treatment. Data were normalized and expressed as the means ± standard error (SEM) of at least three independent experimental runs. Statistical analyses were performed with Graph-Pad Prism 6.01 software (GraphPad Software, Inc.). Unpaired two-tailed Student's t-test or the one-way ANOVA plus Bonferroni post hoc test, were used when comparing two samples or multiple samples, respectively. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of a MF on p53 protein expression levels. The immunoblot analysis revealed that at exposure intervals t >60 min, the MF caused transient changes in p53 expression (Fig. 1A and B), with significant overexpression observed at 90 min (54.40±18.30% over the controls) followed by underexpression at 120 min (73.40±9.70% of the controls). In addition, the RT-qPCR analysis revealed significant overexpression of p53 mRNA (18.90±3.30% over the controls) in samples exposed to the MF for 90 min (Fig. 1C), which is consistent with the overexpression of p53 transcription factor revealed by immunoblotting at the same time interval.

Effect of a MF on the number of cells expressing p53. The immunocytochemical results, summarised in Fig. 2, revealed that a 90-min exposure to a MF significantly increased the rate

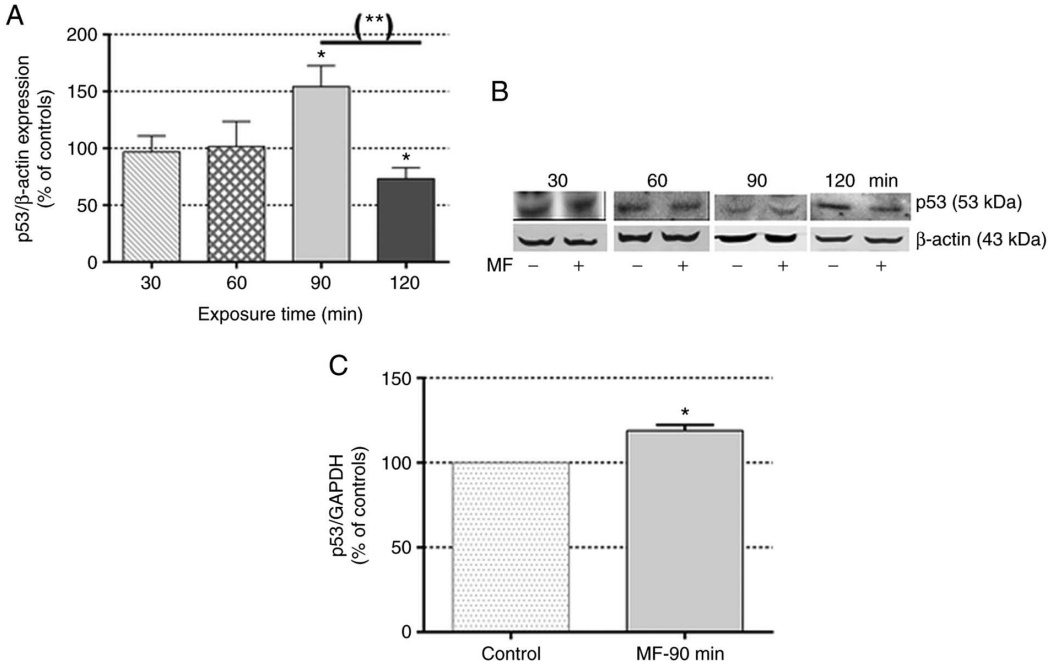

Figure 1. Expression of p53 protein at various MF exposure intervals and p53 mRNA after 90-min MF exposure. (A) Western blot quantification of p53. The data, normalized over the corresponding sham-exposed controls, are the means ± SEM of 4 experimental replicates per interval, with 8 samples (4 MF-exposed and 4 sham-exposed controls) per replicate. *P<0.05 and **P<0.01 (unpaired Student's t-test). (B) Representative blots of p53 at the various exposure (MF +) or sham-exposure (MF -) intervals, using β-actin as loading control. (C) RT-qPCR quantification of p53 mRNA expression using the GAPDH housekeeping gene as a reference. The data, normalized over the corresponding controls are the means ± SEM of 3 experimental replicates with 6 samples (3MF- and 3 sham-exposed per replicate). *P<0.05 and **P<0.01 (one-way ANOVA and Bonferroni post hoc test). MF, magnetic field; RT-qPCR, reverse transcription-quantitative PCR.

of p53-positive cells (p53^{+}; 13.40±0.76% over the sham-exposed controls), while after 120 min of exposure there was a significant decrease in the rate of p53^{+} cells (81.09±1.28% of that in the controls). These results are consistent with those obtained by immunoblotting and RT-qPCR analysis on samples exposed to the field during the same interval.

Effect of a MF on the number of cells expressing wt or unfolded p53. The results of the immunocytochemical analysis of the number of cells expressing p53 protein after 120 min of MF exposure is presented in Fig. 3. Two types of antibodies: PAb1801, which recognizes wt p53, and Pab240, which recognizes the unfolded isoform of p53 but not wt p53, were used to analyze the cellular distribution (nuclear or cytoplasmic) of these isoforms. The results summarized in Fig. 3A revealed that both isoforms were present in control cells, with rates of 65.26±2.5 and 15.10±1.7% for wt p53 and unfolded p53, respectively. MF exposure induced underexpression of wt p53 (18.9±1.3% below the controls) and increased the expression of the unfolded isoform (54.1±6.3% above the controls). These results are consistent with the corresponding immunoblotting data.

The decrease in the rate of wt p53^{+} cells was located exclusively in the cytoplasm (76.26±2.16% of that in the controls; Fig. 3B), with no significant changes in nuclear labeling. By contrast, the increased labeling of unfolded p53^{+} (Fig. 3C) was located both at the nuclear and cytoplasmic levels (56.6±6.1 and 47.92±12.5% over the controls, respectively). The photomicrographs in Fig. 3D and E illustrated these MF effects on labeling distribution.

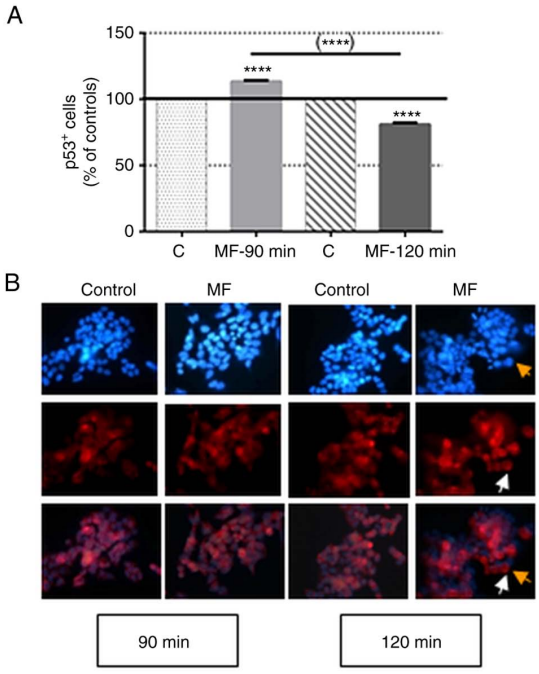

Figure 2. Immunocytochemical analysis of the effects of a MF on the number of cells expressing p53 (p53^{+}). (A) Percentage of p53^{+} cells after 90-min or 120-min exposure. The data, normalized over the corresponding controls, are the means ± SEM of 4 experimental replicates per exposure interval, with 3 MF- and 3 sham-exposed samples per replicate and 15 microscopic fields analyzed per sample; ****P<0.0001 (unpaired Student's t-test); (****)P<0.0001 (one-way ANOVA and Bonferroni post hoc test). (B) Representative images of p53 expression. Upper panels: Cell nuclei were stained blue with Hoechst (yellow arrow); middle panels: p53 expression (red labeling, white arrow); lower panels: Merged images. Magnification, x400. MF, magnetic field.

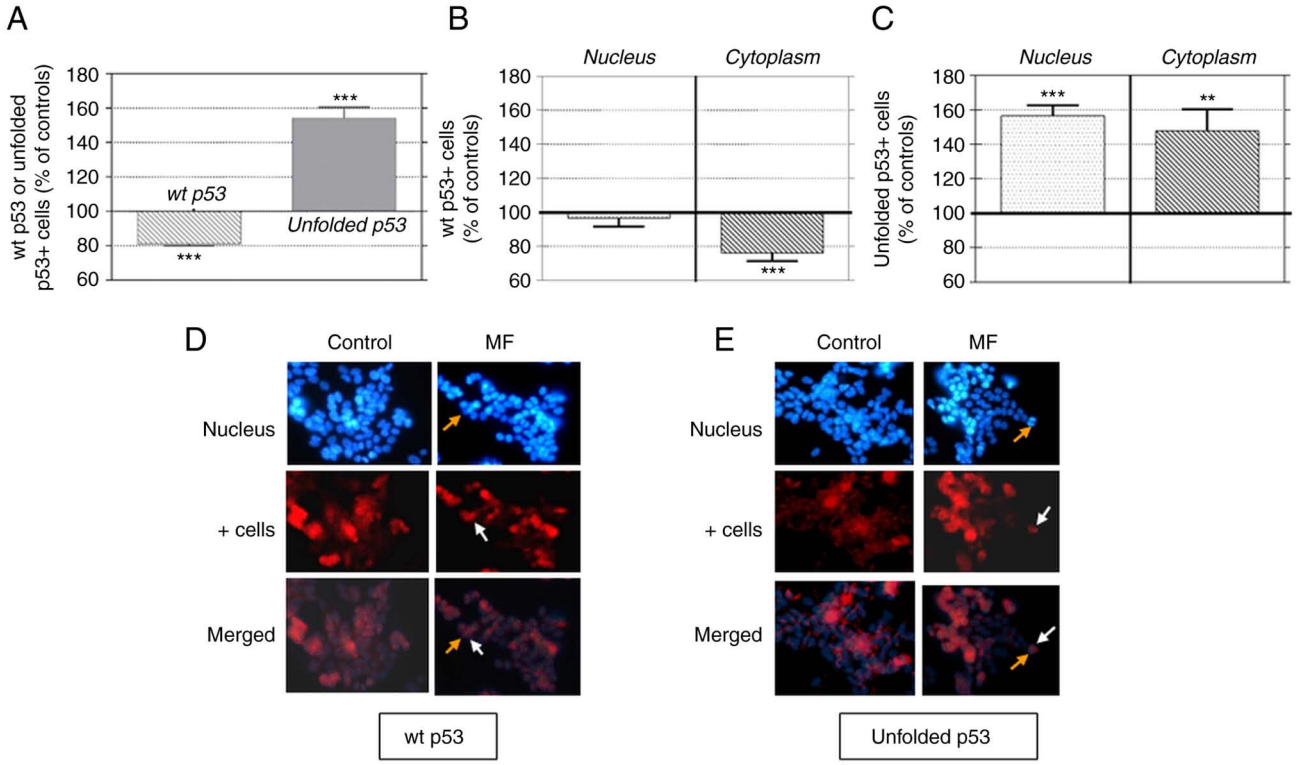

Figure 3. Effects of 120-min of MF exposure on the number of cells expressing p53. (A) The effects of a MF on the number of wt p53^{+} or unfolded p53^{+} cells. (B) The effect of a MF on the nuclear/cytoplasmic distribution of wt p53 labeling. (C) The effect of a MF on the nuclear/cytoplasmic distribution of the specific antigen for unfolded p53. The data, normalized over the corresponding controls, are the means ± SEM of 4 experimental replicates with 3 MF- and 3 Sham-exposed samples per replicate. **P<0.01 and ***P<0.001 (unpaired Student's t-test). (D and E) Representative images of wt p53 and unfolded p53 expression. Upper panels: Cell nuclei were stained blue with Hoechst (yellow arrow); middle panels: p53 expression (red labeling, white arrow); lower panels: Merged images. Magnification, x400. MF, magnetic field; wt, wild-type.

Effects of a MF on Bcl-2 expression. The immunoblotting results revealed that 60 and 90 min of MF exposure induced equivalent increases (35.20±11.40 and 39.20±8.20% over the corresponding controls, respectively) in the levels of Bcl-2 expression (Fig. 4A and B). The immunocytochemical analysis confirmed this effect (Fig. 4C and D), revealing significant increases over the controls in the number of Bcl-2^{+} cells after MF exposure periods of 30 min (19.1±3.6% over the controls), 60 min (27.4±1.4%), 90 min (27.0±1.6%) and 120 min (28.6±2.5%).

Discussion

In previous studies by the authors (38,39,44,45,52) it was demonstrated that intermittent exposure to a 50-Hz, 100-μT MF caused significant changes in the regulation and kinetics of the cell cycle and in the proliferation of human neuroblastoma cells NB69, and these responses were mediated by activation of the EGF receptor and of the signaling pathways MAPK-p38 (free radical dependent) and MAPK-ERK1/2 (free radical independent). These findings, together with the fact that MF exposure increases the overall level of free radicals (40), supports the hypothesis that the effects of the field are partly caused through free radical generation. The present study investigated the possibility that protein p53, which acts as a nuclear transcription factor sensitive to oxidative stress and, in addition to regulating cell cycle control, promotes DNA repair (22), is one of the targets of the ELF field. In control cells, the protein wt p53 exhibits low expression levels due to its degradation through the ubiquitin-mediated proteasome pathway. However, under stress conditions that lead to DNA damage, p53 becomes stabilized and translocates to the nucleus, where it accumulates. This leads to transcriptional activation of several p53 target genes, including those encoding proteins p21WAF1, BAX, and Mdm2, that can result in cell cycle arrest and cell differentiation, senescence, or apoptosis (53,54). Therefore, wt p53 plays a key role in the suppression of cell proliferation control and in tumorigenesis. Thus, a potential, MF-induced dysregulation or loss of p53 activity could alter cell proliferation control mechanisms and eventually trigger processes that can evolve into tumorigenesis.

The results of the present study revealed that in the short term, MF exposure induced a transient effect on the expression levels of wt p53 protein. Indeed, after 90 min of exposure, overexpression of p53 gene and protein were observed, followed 30 min later by significant underexpression of the wt isoform. This underexpression occurred at the cytoplasmic domain and coincided with significant nuclear and cytoplasmic overexpression of the unfolded isoform of the protein.

Previous studies have also reported ELF MF-induced changes in p53 expression, but in general relatively high magnetic flux densities were applied in combination with chemical or physical agents such as ciplastin (55) or X-rays (56). Other studies have reported no changes in p53 expression in cardiac cells exposed continuously (60 min) or intermittently (75 min) to a weak MF of 100 μT at 50 Hz (57).

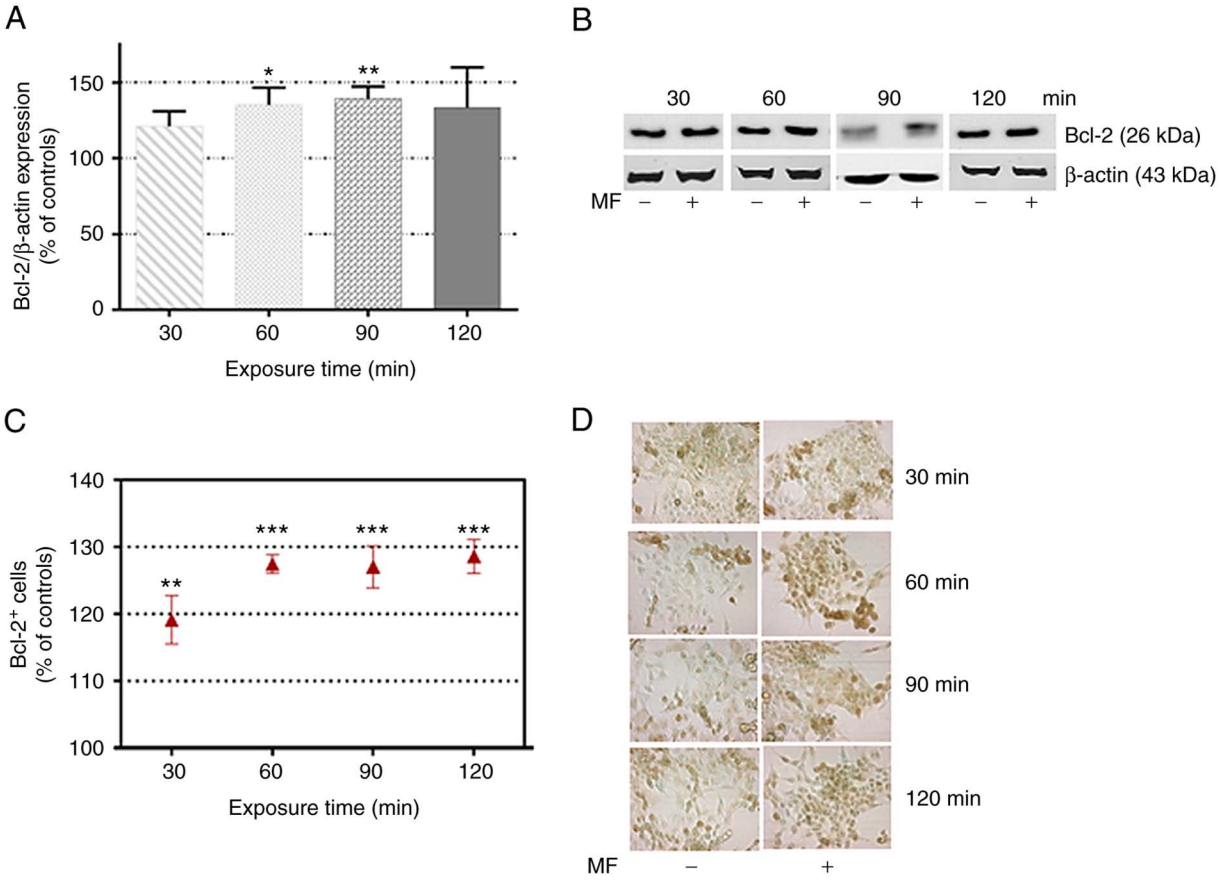

Figure 4. Effects of a MF on Bcl-2 expression. (A) Immunoblot analysis of Bcl-2 expression after 30, 60, 90 or 120 min of MF exposure. Data, normalized over controls, are the means ± SEM of 4 experimental replicates per exposure time, with 3 MF- and 3 sham-exposed control samples per replicate. (B) Representative blots for each of the tested intervals; β-actin was used as loading control. (C) Immunocytochemical analysis of the number of Bcl-2$^+$ cells after 30, 60, 90 or 120 min of MF exposure. Each point represents the mean ± SEM of 4 experimental replicates, with 3 MF- and 3 sham-exposed coverslips per replicate. A total of 15 microscopic fields per coverslips were analyzed. (D) Representative images (magnification x400) of the expression of the antiapoptotic protein Bcl-2 (brown labeling). Nuclei were counterstained with methyl green. *P<0.05, **P<0.01 and ***P<0.001 (unpaired Student's t-test). MF, magnetic field.

In the present study, short exposure (t <90 min) to MF parameters equivalent to those used by these previous authors did not induce significant changes in p53 expression, whereas longer exposure lapses (t ≥90 min) did. Collectively, these data add to the body of evidence suggesting that the exposure cycle and protocol, as well as other physical or biological parameters, can critically influence the type and magnitude of the MF-induced response (58,59).

Conversely, p53 plays a fundamental role in the control of tumor formation. Indeed, the inactivation of p53 generally caused by TP53 gene mutations followed by loss of functionality through a variety of non-mutational regulatory failures, such as alterations in intracellular protein location, is a common phenomenon reported in >50% of various human cancers (60,61). In neuroblastomas and other human primary tumors, this dysfunction is mediated by cytoplasmic sequestration and nuclear exclusion of the wt protein (31,62).

The analysis of the present results does not allow to determine whether the observed effects could be mediated by potential MF-induced DNA alterations. However, the data revealed that field exposure is capable of altering the wt p53 gene and protein expression in the short term and inducing an overexpression of the unfolded isoform of p53, which could contribute to the subsequent proliferative effect observed in NB69 cells after longer exposure intervals to identical MF parameters: 50 Hz and 100 μT (38,39,44,44,52). Such overexpression of the unfolded form of the protein could result from MF-induced alterations in the conformation of the malleable and conformationally labile, pAb240-reactive wt isoform, as it occurs in response to chemical stressors (63). Indeed, it has been reported that *in vitro* stimulation with serum, changes wt p53 to a mutant conformation in murine fibroblasts (64) and that the formation of heterooligomers of the wild and mutated types of p53 could lead to unfolding of the wt protein (65). Therefore, it is possible that some of the aforementioned mechanisms have intervened in the field-induced conformational changes of p53 that manifest as overexpression of the unfolded isoform.

In addition, there is experimental evidence indicating that mutations and defects in the folding of p53 disable this protein from exerting its tumor suppressive functions in cancer cells, while enabling it to actively intervene in various stages of tumor progression and to promote resistance to anticancer treatments (66,67). The present results indicate that, in addition to inducing p53 gene and protein overexpression, MF exposure could cause alterations in protein folding that are likely to intervene in the field-induced proliferation promotion of neuroblastoma cells.

On the other hand, the tumor suppressor protein p53 functions as a stress-sensitive transcription factor, and there is compelling evidence that ELF-MFs affect cell physiology by altering redox-related processes (59,68). Specifically, in human neuroblastoma NB69 cells, exposure to a 100-μT MF at 50 Hz was revealed as a stress factor (38,40) capable of increasing free radical levels and of dysregulating mitogenic stress transduction and p53 regulation pathways, such as the Jun NH2-terminal kinase pathway (69,70). Under stress conditions, both wt and mutated p53 accumulate in the cell, and only wt p53 returns to basal concentration levels once normal conditions are restored. The fact that the levels of mutant p53 remain elevated is attributed, at least in part, to lack of an autoregulatory loop of wt p53, mediated by Mdm2 and other negative regulators (61). A similar effect could be involved in the presently reported transient overexpression or accumulation of wt p53 induced by the MF, and in the subsequent underexpression of the wild form and overexpression or accumulation, both at the nuclear and cytoplasmic levels, of the unfolded isoform of the protein. Therefore, as a whole, these data suggest that the alterations observed in the expression of both conformations of p53 could result from a MF-induced redox modification of the protein, and that such alterations could mediate the subsequent NB69 cell cycle dysregulation and cell proliferation promotion reported in the aforementioned studies (38,39,44,45,52).

These results also revealed that a 100-μT MF at 50 Hz causes transitory overexpression of the antiapoptotic protein Bcl-2 at 60 and 90 min of exposure. Other authors have also reported MF induction of Bcl-2 overexpression in various cell types including MCF-7 (71), CHO-K1 (72), xrs5 (56) or leukemia K562 (73). However, those studies applied higher density MF (1-5 mT) in combination with ionizing radiation (X-rays; 1-12 Gy) or chemical agents. In most cases, exposure to the MF inhibited the apoptosis induced by physical or chemical agents, which resulted in overexpression of Bcl-2, an increased number of cells in the G1 phase, and inhibition of p53 expression.

In addition to acting as a nuclear transcription factor, wt p53 can directly interact with cytoplasmic proteins such as Bcl-2, and neutralize their anti-apoptotic activity (34,35). The MF-induced alterations in p53 described in the present study could affect such an interaction and result in the observed overexpression of free Bcl-2, which is capable of triggering antiapoptotic and/or cell survival-promoting responses, as reported in previous studies on NB69 cells exposed for longer periods to the same MF stimulus (38,39,44,45,52).

In conclusion, the present results demonstrated that exposure to a 50-Hz and 100-μT MF causes increased expression of Bcl-2, accompanied by early overexpression (at 90 min of exposure) of the TP53 gene and transient overexpression of the wt of the corresponding protein. This transitoriness would be caused by overexpression of the unfolded configuration of the p53 protein and the corresponding underexpression of the wt p53 configuration, both observed at 120 min of MF exposure. Such alterations could affect the functions of p53 in the cytoplasmic and nuclear domains, thus mediating the MF-induced dysregulations of DNA synthesis, the cell cycle and cell proliferation in NB69 cells that have been described in previous studies by the authors.

These results build on those of previous studies (38,39,44, 45,52) and provide complementary information affording a more complete picture of the cascade of effects involved in the proliferative response of the NB69 cell line. These studies are not intended to resolve current controversies about the potential carcinogenicity of chronic exposure to weak ELF fields, nor are they intended to be applied for the evaluation of real risk levels in cases of carcinogenicity. Their purpose is to characterize the mechanisms underlying the cell response to a physical stimulus that current safety standards adjudge too weak to be biologically relevant.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MAM and MAT designed the study, conducted the experiments, analyzed the data and wrote the manuscript. AU designed the study, analyzed the data and also participated in the writing of the manuscript. JMB conducted the RT-qPCR analyses and also participated in the writing of the manuscript. MAM and MAT confirm the authenticity of all the raw data. All authors read and approved the manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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