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Original Paper

Involvement of the EGF Receptor in MAPK Signaling Activation by a 50 Hz Magnetic Field in Human Neuroblastoma Cells

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Key Words
Magnetic fields • ELF • NB69 cells • EGFR • ERK1/2

Abstract
Background/Aims: Previous studies have shown that a 63-hour, intermittent exposure to a 50 Hz, 100 μT magnetic field (MF) induces in the NB69 line of human neuroblastoma a proliferative response that is mediated by activation of the MAPK pathways ERK1/2 and p38. The present study aims to investigate the potential involvement of the epidermal growth factor receptor (EGFR) in the field-induced cell proliferation and activation of MAPK pathways. ***Methods:*** NB69 cultures were MF- or sham-exposed for 5 to 30 minute intervals and 63 hours. Cell proliferation and activation of MAPK-ERK1/2, -p38 and -JNK was analyzed in the presence or absence of erlotinib, an effective inhibitor of EGFR tyrosine kinase. The expression of p-EGFR and MMP-9 in the presence or absence of MF was also studied. Between 3 and 7 replicates of each experiment were performed, using between 3 and 4 samples per experimental condition and replicate. At the end of each replicate, the samples were analyzed at short times (5-30 min) through immunofluorescence and Western blotting, and the growth response was assessed (63 hours interval) through dye exclusion with Trypan blue. ***Results:*** The results confirmed that field exposure induces cell proliferation and activation of ERK1/2, p38 and JNK, and revealed that these effects were blocked with erlotinib. The data also showed that, compared to sham-exposed controls, the MF exposure induces early and transient increases in the expression of p-EGFR and MMP-9 at 15 and 5 min from the exposure onset, respectively. ***Conclusion:*** The obtained results reveal that the activation of the MAPK-ERK1/2 and -p38 pathways by the MF is mediated by the EGF receptor. Taken together with our previously published results, this dataset suggests that the proliferative response induced in NB69 by a 63-hour exposure to a weak, power frequency MF, is mediated by early transient activation of EGFR in which MMP-9 would be involved.

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Introduction

Due in part to the widespread exposure of the general population to magnetic fields (MF) emitted by systems related to the generation, distribution and use of electricity, a debate has been going on for years focusing on the possibility that exposure to power frequency (50-60 Hz) fields could have adverse health effects, being those related to cancer development and/or promotion the most widely studied ones. Although the evidence of an association between chronic occupational exposure to these extremely low frequency (ELF) MF and the incidence of cancer is limited [1-3], the International Agency for Research on Cancer (IARC) of the World Health Organization, included this type of physical agent in its list of possible carcinogens, class 2B (IARC 2002 [4]), mainly on the basis of the epidemiological link between incidence of childhood leukaemia and residential exposure to MF [5-7].

Regarding the biomechanisms underlying a potential carcinogenic action, given that ELF MF lack sufficient energy to directly cause DNA damage, it is postulated that exposure to such fields could affect the epigenetic cell regulation, which is known to be susceptible to a variety of environmental factors, including exposure to non-mutagenic carcinogens [8]. In fact, although the results of some experimental studies are not supportive of the potential carcinogenic effects of weak ELF fields [9-11], others have reported that these fields are capable of inducing significant alterations in DNA repair or stability [12-14], as well as in cell proliferation and differentiation [15-17].

Regarding cell proliferation, we have reported that intermittent, 3 h On/3 h Off, exposure to 50 Hz magnetic fields at 10 - 100 μT increases cell proliferation in the hepatocarcinoma HepG2 and neuroblastoma NB69 human cancer lines [18-20]. In neuroblastoma, at 15 - 30 minutes after onset of the 3-hour MF exposure intervals, significant activation was recorded of signaling pathways MAPK-ERK, -p38 and -JNK, being the subsequent proliferative effect of the field mediated by joint activation of the signal transduction pathways MAPK-ERK1/2 and -p38 [21, 22]. Other authors have reported that short-term (30 min) exposure to ELF MF can also induce ERK activation in different biosystems, including transformed and non-transformed cells [23-26]. Consequently, and knowing that the cascade of MAPK-dependent intracellular phenomena intervenes in the gene regulation of cancer cell proliferation, differentiation and survival, it is conceivable that such signaling cascade plays a critical role as an epigenetic process underlying the mitogenic effects of ELF fields.

Also membrane receptors have been proposed to be among the most relevant targets for MF. For instance, exposure to dynamic magnetic fields ranging from ELF to radiofrequency has been shown to significantly influence the activity of the epidermal growth factor receptor (EGFR) [27-29].

On the other hand, the EGFR and its signaling pathways, including MAPK, are involved in the modulation of proliferation, both in normal physiological processes and in tumor development [30-32]. In fact, the expression of the phosphorylated form of EGFR (pEGFR) contributes to a malignant phenotype [33] and is correlated with poor prognosis [34]. In addition, repression of the activation of EGF receptors by erlotinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, potently suppresses growth in various tumors [35]. On the basis of this set of evidence, the EGFR can be postulated as a potential target in the proliferative response to ELF fields, even if the current knowledge on the molecular mechanisms underlying the involved processes remains insufficient.

There are also indications that matrix metalloproteinases (MMP) could mediate the cellular response to MF, as observed by Patruno et al. (2012) [25], who reported that exposure to a 50-Hz, 1.0 mT field can increase MMP2 and MMP-9 activities in THP-1, a monocytic cell line of human leukemia. MMP degrade the extracellular matrix and exert key functions in cancer injury and regeneration. Specifically, gelatinase MMP-9 has been reported to play potential pro-oncogenic roles, intervening in neoplastic transformation, tumor initiation/promotion and genetic instability [36]. It is therefore conceivable that MMP can mediate in some of the processes leading to the proliferative response of cancer cells to ELF MF.

The present study investigates the effects of the exposure to a 50 Hz, 100 μT MF on EGFR receptor activation and MMP-9 expression in NB69 cells, as well as the potential involvement of the EGFR receptor in the previously reported proliferation and activation of MAPK-ERK1/2, -p38 and -JNK induced by the same MF [21, 22]. The obtained results show that inhibition by erlotinib of the EGFR activation results in blockade of the MF-induced cell proliferation and activation of MAPK pathways. This strongly suggests that a precocious MF effect on the EGFR mediates in the subsequent, field-induced activation of MAPK- ERK1/2 and -p38 signaling, which would lead to the reported increases in neuroblastoma cell proliferation [19-22].

Materials and Methods

Cell culture

Cells from the human neuroblastoma line NB69, obtained from the European Collection of Authenticated Cell Culture (ECACC, Salisbury, UK) were seeded in 60 mm plastic Petri dishes (Nunc, LabClinics, Barcelona, Spain) containing Dulbecco's Minimum Essential Medium (DMEM Biowhittaker, Lonza, Verviers, Belgium) supplemented with 10%, heat inactivated foetal bovine serum (FBS, Gibco BRL, Invitrogen, Paisley, Scotland, UK), 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 0.25 μg/ml of amphotericin B as antimycotic agent (Gibco BRL,Thermo Fisher Scientific, Waltham, MA USA) and were grown for 4 days in a 5% CO_2, humidified atmosphere inside a CO_2 incubator (Thermo Fisher Scientific). In each experimental run 4.5×10^4 cells ml^{-1} were seeded either directly on the bottom of the Petri dishes or on 12-mm diameter glass coverslips placed inside the dishes.

Treatment with inhibitor of epidermal growth factor receptor

On day four after seeding, and 1 hour prior to the MF- or sham-exposure onset, the medium was renewed and supplemented with EGFR inhibitor or with the corresponding vehicle. The treated samples received 1 μM erlotinib (BioSource, B-1400, Nivelles, Belgium), a reversible inhibitor of EGFR tyrosine kinase that binds competitively to the ATP-binding site of the receptor's kinase domain. The inhibitor was reconstituted in DMSO (Sigma-Aldrich, St. Louis, MO, USA) at a stock concentration of 1 mM. An equal volume of DMSO, not exceeding 0.1% of the volume of the media, was added to the unexposed samples.

Magnetic field exposure

The cultures were exposed to a 50 Hz, sine wave, vertically polarized MF, at a magnetic flux density B_{AC} = 100 μT root mean square (rms). The exposure set-up and procedure have been described elsewhere [19, 21]. Briefly, current flow was supplied by a wave generator (Newtronic Model 200MSTPC, Madrid, Spain) having a 3.53 mA DC offset (B_{DC} = 15 μT rms). The generator was connected to a pair of coils set in Helmholtz configuration. The current in the coils was monitored using a multimeter (Hewlett Packard, model 974A, Loveland, CO, USA) and the induced MF was routinely checked with two magnetometers (EFA-3, Wandel and Goltermann, Eningen, Germany, and EMDEX II, Enertech Consultants, Campbell, CA, USA). One Helmholtz coil pair was placed inside each of two magnetically shielded chambers (co-netic metal; Amuneal Corp., PA, USA) located within two identical CO_2 incubators (Thermo Fisher Scientific). The background MF inside the shielded chambers was B_{AC}: 0.04 ± 0.03 μT (rms); B_{DC}: 0.05 ± 0.04 μT (rms). No increase of temperature at the samples location was observed using two pt100 thermocouple probes (Fluke, Model 52, Adler Instruments, Madrid, Spain) when the coils were energized to produce the desired magnetic flux density of 100 μT rms. In each experimental run Petri dishes containing the cell samples (5 dishes per experimental group) were stacked in the central region of the Helmholtz coil gap, in which uniformity of MF exposure is ensured. Following a random sequence, only one of the two identical coil sets was energized in each experimental run, being the samples located in the unenergized set considered sham-exposed controls. In order to ensure sufficient cell density to allow proper analysis of the short-term response, the MF and sham treatments were applied simultaneously at day 4 post-plating. The effects of the MF on the expression/activation of selected transduction pathways were studied after short exposure intervals ranging 5 to 30 min.

Proliferation assay for growth response after 63 hours of field exposure in the presence or absence of the inhibitor erlotinib

In each of 4 experimental replicates NB69 cells were plated on 20 Petri dishes: 10 dishes were treated with 1 μM erlotinib, and 10 with vehicle. Sixty minutes after, the samples were distributed in each of the two sets of coils located in shielding chambers inside the incubators, and submitted for 63 h to the following treatments: (1) sham exposure in the absence of erlotinib (2) sham exposure in the presence of 1 μM erlotinib; (3) MF exposure only and (4) MF + 1 μM erlotinib. Five Petri dishes were used per experimental condition. On day 6 post-plating, at the end of the 63-hour lapse of intermittent (3 h On/ 3 h Off) field exposure and/or incubation, the samples' cell viability and cell number were assessed through dye exclusion with 0.4% Trypan blue (Sigma).

Immunofluorescence

After MF- or sham- exposure of the samples cultured on coverslips, the activation of EGFR, ERK1/2 and p38, as well as the expression of MMP-9 were characterized by indirect immunofluorescence and computer-assisted image analysis. The coverslips were incubated with primary antibodies against p-ERK1/2 (1:100; 44-680G, Thermo Fisher Scientific), p-EGFR (1:100, 3777, Cell Signaling, Danvers, MA, USA), p-p38 (1:100; 9216, Cell Signaling) and MMP-9 (1:200; EP1254-ab-76003, Abcam, Cambridge, UK). Anti-mouse-IgG conjugated to AlexaFluor® 546 or anti-rabbit IgG conjugated to AlexaFluor® 488 (Molecular Probes, Eugene, OR, USA) secondary antibodies were used to reveal the proteins of interest. The nuclei were counterstained by Hoechst 33342 (Bisbenzimide, Sigma-Aldrich) added to the mounting medium. Fluorescent images, captured using a Nikon Eclipse TE300 inverted microscope, were analyzed by analySIS 3.0 image-analytical software (GMBH, Münster, Germany). In each of 4 experimental runs, 4 coverslips were studied per each of 4 experimental groups: Sham-exposed controls, MF-exposed in the presence of erlotinib, MF-exposed only, erlotinib only. Fifteen microscope-fields per coverslip were randomly selected and photographed for image analysis. The total number of nuclei and the percent of p-EGFR, p-ERK1/2, p-p38 and MMP-9 positive cells per microscope field were recorded.

Western blotting analyses

The total protein extraction and immunoblotting procedures have been described elsewhere [21]. Briefly, after MF- or sham-exposure the cells were lysed in hypotonic lysis buffer and the protein content of the lysates was quantified by Bradford's assay. To do so, equal protein volumes from each of the samples were separated using 10% or 8% SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membranes were incubated with a primary antibody against p-EGFR (1:1000; Cell Signaling), p-ERK1/2 (1:1000; Thermo Fisher), p-p38 (1:1000; Cell Signaling), p-SAPK/JNK (1:1000; 4668, Cell Signaling) and MMP-9 (1:1000; Abcam). Anti-human β-Actin (1:5000; A5441, Sigma-Aldrich) was used as loading control in all experiments. After incubation with the indicated primary antibodies, the detection of the proteins of interest was performed using peroxidase-conjugated secondary antibodies (GE Healthcare) or fluorescently labelled secondary antibody IRdye (LI-COR, Bioscience, Lincoln, NE, USA). The blots were revealed by ECL-chemiluminescence on a ProXima imaging system (Isogen Life Science Veldzigt, Utrecht, Netherlands) or by fluorescence signal detection and quantification with Odyssey infrared imaging system (LI-COR). Equal protein loading was confirmed by β-Actin (Sigma-Aldrich) immunoblot. For semiquantitative immunoblot analysis, the optical density of the bands was measured and quantified by densitometry through computer imaging software (Quantity-One, BioRad, Munich, Germany). At least four experimental replicates were conducted for each of the studied proteins. Three MF-exposed and three sham-exposed dishes were used per experimental run and exposure interval.

Statistical analysis

All experimental procedures and analyses were conducted blindly for treatment. Data were normalized and expressed as means ± standard error (SEM) of at least three independent experimental runs. Statistical analyses were performed using Graph-Pad Prism 6.01 software (GraphPad Software, Inc., La Jolla, CA, USA). Two-tailed student's *t*-test and one-way ANOVA test were applied when comparing 2 samples or multiple samples, respectively. The limit of statistical significance was set at $p < 0.05$.

Results

Effects of the chemical inhibition of EGFR on MF-induced cell proliferation

The data in Fig. 1 show a significant increase in the number of NB69 cells (21.3% over controls) in samples exposed intermittently to the MF for 63 hours, which confirms the cytoproliferative action of the field described in previous studies [19, 20]. In the absence of MF exposure, the inhibitor of EGFR phosphorylation, erlotinib, induced a significant decrease in cell number (12.04% below that in vehicle-treated controls). Exposure to MF in the presence of the inhibitor had an antiproliferative effect (13.68% cells below the corresponding controls) equivalent to that obtained when the inhibitor was administered in the absence of MF. These results are indicative of a potential involvement of the EGF receptor in the proliferative response of NB69 to the MF.

Time-dependent activation of the EGF receptor by the MF

The expression of the active form of the EGF receptor was analyzed at the end of short intervals of MF exposure. The immunocytochemical results showed significantly increased rates of p-EGFR positive cells (p-EGFR+) after 10 and 15 minutes of field exposure, but not at 5 or 20 minutes (Fig. 2 A, B). These results were consistent with those of p-EGFR protein expression levels, which were significantly increased after 10 and 15 minutes of MF exposure, but did not differ from those in sham-exposed controls after 5 or 20 minutes of treatment (Fig. 2 C, D).

Effects of the chemical inhibition of EGFR on the response of p-ERK1/2 expression to the MF

The field effects on the p-ERK1/2 expression levels in the presence or absence of erlotinib were analyzed. MF exposure intervals of 20 and 30 minutes were selected on the basis of previously reported data on the effects of the same exposure parameters on p-ERK1/2 expression [18]. The immunocytochemical results showed significantly increased rates of p-ERK1/2+ cells in the samples treated with the MF during both of the exposure intervals, this response being inhibited by the presence of erlotinib (Fig. 3 A, B). These results were reinforced by those of the Western blotting analysis, which showed significant p-ERK1/2 overexpression, both at 20 and 30 minutes of MF exposure, which was blocked by erlotinib (Fig. 3 C, D).

Effects of the chemical inhibition of EGFR on the response of p-p38 and p-JNK expression to the MF

As the activation of MAPK-p38 and -JNK pathways has been shown to occur short (15 min) after the field exposure onset, and prior to MAPK-ERK activation [19], in the present study the MF effects on the rate of p-38 positive cells and on the expression of phosphorylated forms of p-38 and p-JNK were analyzed only at 20 minutes of exposure. The results illustrated in Fig. 4 (A, B) show that at the end of that interval, the rate of activated, p-p38+ cells was significantly increased, this MF effect being inhibited when erlotinib was present in the medium. Similarly, the MF-induced overexpression of the phosphorylated forms of proteins p38 and JNK (p-p38 and p-JNK) did not occur in the presence of the inhibitor (Fig. 4 C, D and Fig. 5 A, B).

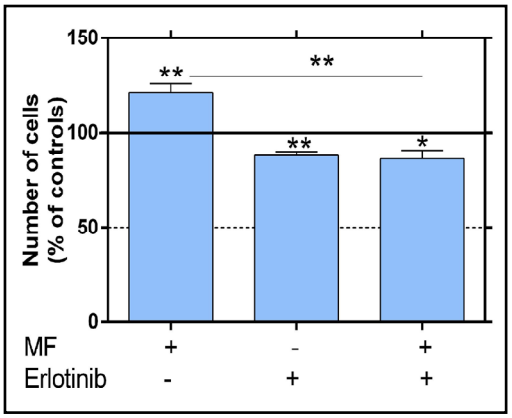

Fig. 1. Number of viable cells after 63 h of intermittent exposure (3 h On/3 h Off) to the MF, in the presence of 1 μM erlotinib or 0.0 μM (vehicle). The data, normalized over the corresponding controls, are means ± SEM of four experimental replicates. *: $0.01 \leq p<0.05$; **: $0.001 \leq p<0.01$ (ANOVA and Student's t test).

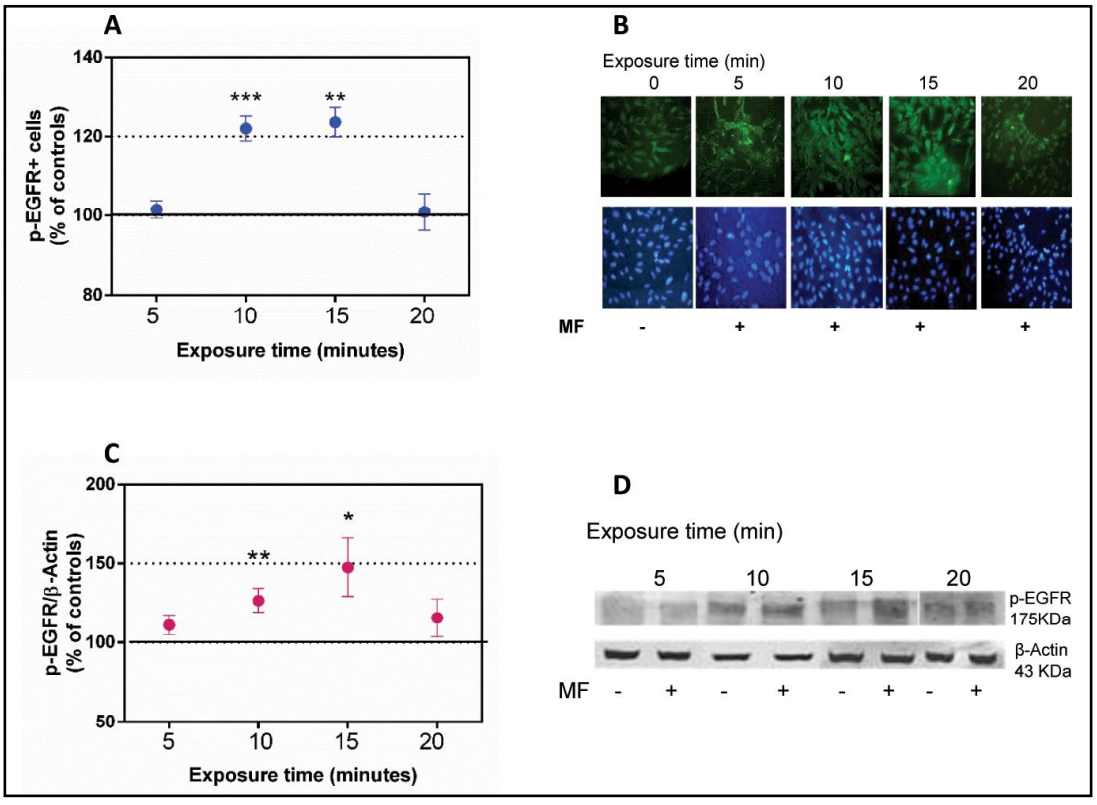

Fig. 2. Rate of phosphorylated EGFR-positive cells (p-EGFR+) and Western blot quantification of p-EGFR expression, after different MF- or sham-exposure intervals. (A) Immunocytochemical and computer-assisted image quantification of the rate of p-EGFR+ cells. Each point represents the mean ± SEM of 4 experimental replicates, with 8 samples (4 MF- and 4 sham-exposed) per replicate. Values are normalized over controls (line 100%) **: 0.001 ≤ p<0.01; ***: p<0.001 (ANOVA and Student's t-test). (B) Upper panel: Representative images of p-EGFR labeling at the time intervals assayed. Lower panel: Hoechst-stained nuclei of the cells in the corresponding upper micrographs. MF-: sham-exposed controls; MF+: samples exposed to the MF for 5 to 20 minutes. (C) Western blot quantification of p-EGFR expression using β-Actin as load control. The values, normalized over the corresponding sham-exposed controls, are means ± SEM of at least 4 experimental replicates per exposure time, with 6 samples (3 MF- and 3 sham-exposed) per replicate. A total of 114 samples were analyzed. *: 0.01 ≤ p<0.05; **: 0.001 ≤ p<0.01 (Student's t-test). D) Representative blots at the different exposure (MF+) or sham-exposure (MF-) intervals.

MF effects on the expression of Metalloproteinase-9

The extracellular metalloproteinase MMP-9 plays an indirect role in cell proliferation regulation and is potentially involved in activation of the EGF receptor during carcinogenic processes [36, 37]. The immunocytochemical results revealed a significant increase in the rate of cells expressing MMP-9 at 5 minutes of MF exposure, but not at longer exposure intervals, of 10 and 15 minutes (Fig. 6 A, B). Similar results were obtained by Western blotting analysis, which showed a significant increase in MMP-9 protein expression at 5 minutes, but not at 10 or 15 minutes of field exposure (Fig. 6 C, D).

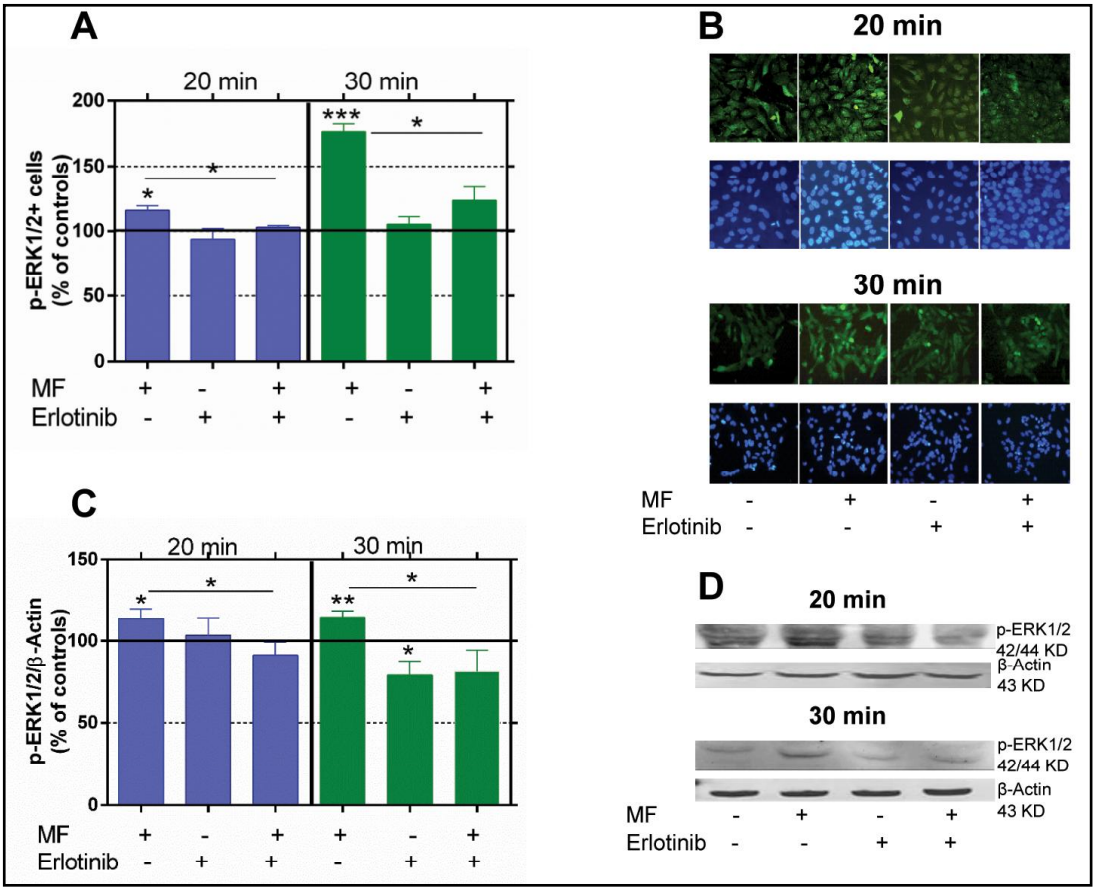

Fig. 3. Effects of 20- or 30-minute MF exposure on the rate of p-ERK1/2+ cells and on p-ERK1/2 protein expression, in the presence or absence of the inhibitor of EGFR phosphorylation, erlotinib. Four experimental conditions were assayed: -/-: untreated controls; +/-: MF only; -/+: erlotinib only; +/+: MF plus erlotinib. (A) Immunofluorescence quantification by computer-assisted analysis of photomicrographic images. Means ± SEM of 3 experimental replicates, with 4 samples per replicate and experimental condition. Normalized values.*: 0.01 ≤ p<0.05; ***: p<0.001 (ANOVA and Student's t-test). (B) Upper panel: representative images of p-ERK1/2 labeling (green) at the two studied intervals and under the corresponding experimental conditions. Lower panel: Hoechst-stained nuclei of the cells in the corresponding upper micrographs. (C) Western blot quantification of p-ERK1/2 expression, using β-Actin as load control. The values, normalized over the corresponding sham-exposed controls are means ± SEM of 5 experimental replicates, with 3 samples per replicate and experimental condition. *: 0.01 ≤ p <0.05; **: 0.001 ≤ p <0.01 (Student's t-test). (D) Representative blots.

Discussion

The interaction between extracellular signals and their specific receptors is usually the initial step in signal transduction. In mammals, signaling through the EGFR, a 170 kDa transmembrane tyrosine kinase receptor, is crucial in regulation of cell proliferation, differentiation and survival. On the other hand, there is ample evidence that EGFR also plays a fundamental role in tumor transformation and progression, being overexpression of the active forms of EGFR common in several neuroblastoma and primary tumor cell lines [38]. Besides, abnormal activation of EGFR is often associated with activation of different signaling pathways, such as RAS/RAF/MEK/ERK, PI3K/AKT, Src/p38/YY1 and GBP1, that trigger cascades of cancer cell survival and proliferation, contributing to increase the invasive capacity of these cells [33, 34, 39, 40].

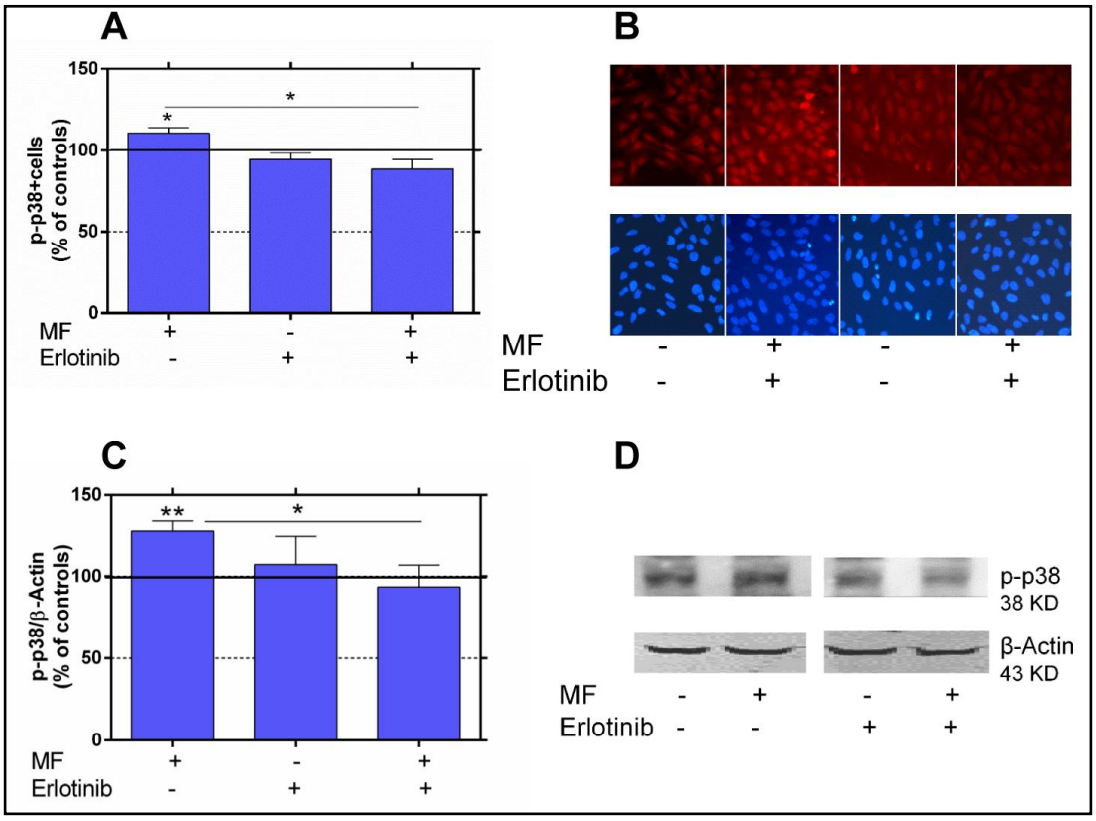

Fig. 4. Effects of 20 min of MF exposure on the rate of p-p38+ cells and on p-p38 protein expression, in the presence or absence of erlotinib. (A) Rate of p-p38+ cells. Immunofluorescence quantification by computer-assisted analysis of photomicrographic images: normalized values. *: 0.01 ≤ p <0.05 (ANOVA and Student's t-test). (B) Upper panel: representative images of p-p38 labeling. Lower panel: Hoechst-stained nuclei of the cells in the corresponding upper micrographs. (C) Western blot quantification of p-p38 expression. β-Actin was used as charge control. Values are normalized over controls. Bars are means ± SEM of 5 experimental replicates, with 3 samples per experimental condition and replicate. *: 0.01 ≤ p <0.05; **: 0.001 ≤ p<0.01 (Student's t-test). (D) Representative blots for p-p38.

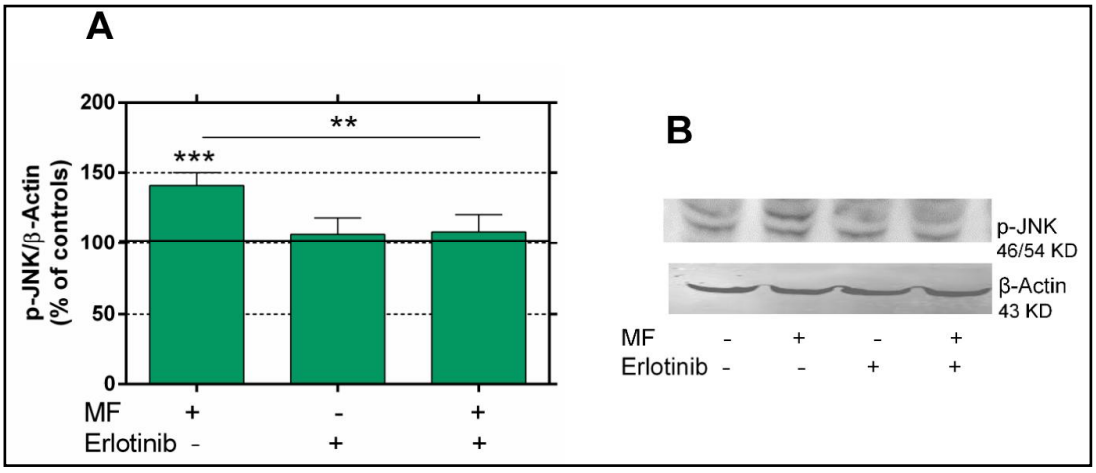

Fig. 5. Western blot analysis of p-JNK expression in samples exposed to the MF for 20 minutes in the presence or absence of erlotinib. β-Actin was used as charge control. Values are normalized over controls. (A) p-JNK expression: bars are means ± SEM of 7 experimental replicates, with 3 samples per experimental condition (control, MF only, erlotinib only, MF+erlotinib) and replicate. **: 0.001 ≤ p <0.01; ***: p<0.001 (Student's t-test). (B) Representative blots.

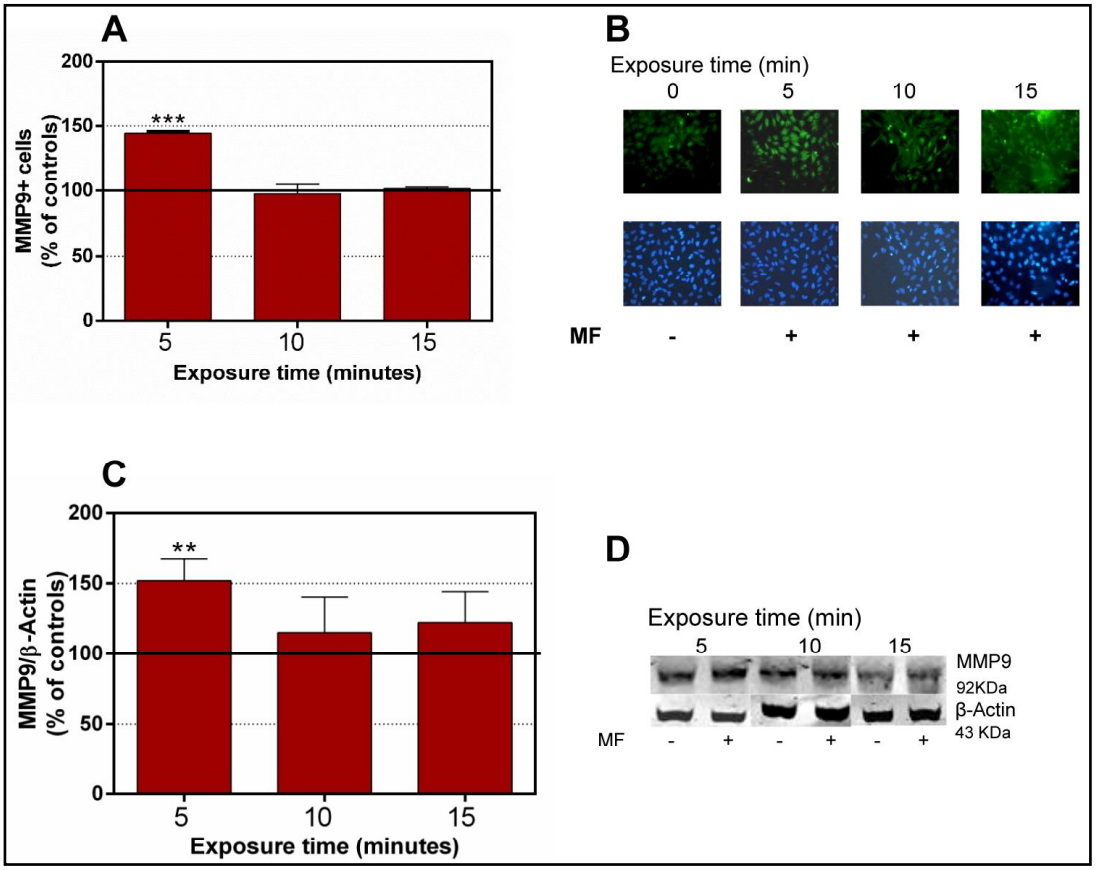

Fig. 6. Rate of MMP-9+ cells and MMP-9 protein expression after 5, 10 or 15 minutes of MF exposure. (A) Immunofluorescence quantification of MMP-9+ cell rate by computer-assisted analysis of photomicrographic images. Bars are means ± SEM of 4 experimental replicates, with 4 samples per experimental condition (sham- or MF-exposure), exposure time and replicate. Data are normalized over controls. ***: p <0.001 (Student's t-test). (B) Upper panel: representative images of MMP-9 labeling in sham-exposed controls (MF-) and in samples exposed to the field (MF+) for -5, -10 or -15 minutes. Lower panel: Hoechst staining of the corresponding nuclei. (C) Western blot analysis of MMP-9 expression, using β-Actin as charge control. Normalized data. Bars are means ± SEM of 6 experimental replicates, with 3 samples per experimental condition (sham- or MF-exposure), exposure time and replicate. **: 0.001 ≤ p<0.01 (Student's t-test). (D) Representative blots at the different time intervals; MF+: field-exposed samples; MF-: sham-exposed controls.

Among the signaling pathways initiated by EGFR activation, RAS/RAF/MEK/ERK, an essential pathway for EGF-mediated cell proliferation, is the best known and best characterized [41]. Other intracytoplasmic signaling pathways, such as p38 and JNK, which are involved in processes of cell proliferation and transformation, as well as in cell migration and resistance to apoptosis, are also activated by EGFR [42, 43].

The present study investigates the potential involvement of EGFR in the proliferation and activation of ERK1/2, p38 and JNK pathways, which in the NB69 cell line have been shown to be responsive to the exposure to a 50 Hz, 100 μT MF [21, 22]. To that end, we first investigated whether the proliferative response of NB69 can be affected by the specific inhibitor of the EGFR activation, erlotinib, whose inhibitory effects have been characterized in different cancer types, including pancreatic cancer, ovarian cancer or non-small cell lung cancer (NSCLC). In fact, erlotiniob has been approved by the US FDA for the treatment of patients with advanced or metastatic NSCLC [44]. Next, we investigated the potential field effects on the activation of EGFR and of MAPK pathways in the presence or absence of erlotinib.

The obtained results reveal that MF exposure causes significant increase in the early and transient activation of EGFR, which peaked at 10 and 15 min of the MF onset, which reinforces previously reported evidence in other cellular models. For instance, in Chinese hamster lung cells, increased formation of EGFR clusters has been described after 5 and 15 minutes of exposure to a 0.4 mT MF [27, 28]. Likewise, in human amniotic cells, increased formation of EGFR clusters and changes in the phosphorylation state of the receptor have been described after 15 minutes of exposure to 50-Hz fields at 0.1, 0.2 and 0.4 mT [29]. Transient activation patterns such as that reported herein, have been described in biological systems exposed to different physical or chemical stress factors [45]. Concerning the pEGFR overexpression at 10 and 15 minutes only, the transient nature of the response could indicate that MF susceptible cells would initially need of a minimum time interval to activate a sufficient number of receptors, perhaps through a clustering action like that observed after 15 minutes of treatment by Ke et al. (2008) and Sun et al. (2008) [28, 29]. The absence of observable effects in later exposure phases could be due to a triggering, either spontaneous or induced by the field itself, of cellular adaptation mechanisms responsible for maintaining low, steady state EGFR levels [46].

Previous works by our group had revealed that signaling pathways MAPK-ERK1/2, p38 and JNK are activated in NB69 by exposure to a 50 Hz, 100 μT MF. Indeed, intermittent exposure induced an early, transient and repetitive activation of ERK1/2 that peaked at 30 minutes from the start of each 3-hour exposure cycle [21]. The MF also induced early and transient activation of p38 and JNK, which reached maximal levels between the 15 and 30 initial minutes of each exposure cycle [22]. Chemical inhibition of ERK1/2 or p38 phosphorylation, reverted the proliferative effect observed at 63 hours of field exposure, indicating that these pathways are involved in the proliferative response of NB69 to the MF. The present results also evidence the implication of EGFR in said mitogenic field effects, as they are blocked by chemical inhibition of EGFR activation. This, together with the observation that a 20-minute treatment with erlotinib blocks the MF-induced activation of ERK1/2 and p38, indicates that the EGFR receptor could mediate the MF-induced proliferative effect, through its action on said signaling pathways.

On the other hand, increased immunoreactivity of the matrix metalloproteinase MMP-9 has been described in neuroblastoma proliferating cells and in bone marrow macrophages [47]. Indeed, MMP-9 exerts a critical function in the regulation of the turnover (disintegration and remodelling) of the extracellular matrix, and its aberrant/unbalanced regulation is functionally correlated with pathological processes, including tumor progression [36, 48, 49]. Results of this study show significantly increased levels of MMP-9 expression and of MMP-9 positive cells, at early phases (5 minutes) of the field exposure, prior to the MF-induced activation of the EGFR (15 minutes). These results are in agreement with those by other authors who reported increased MMP-9 expression in cells of the THP-1 line of acute myeloid leukaemia and in kidney tissues of male Wistar albino rats, exposed to pulse and sine wave, 50-Hz MF at flux densities between 0.1 and 1.5 mT [25, 50].

The expression of MMP-9 has also been related to EGFR activation or overexpression in human carcinoma cells [51]. Moreover, EGFR activation mechanisms mediated by metalloproteinases have been described [52] that could trigger the subsequent activation of MAPK signal transduction pathways [53]. Thus, even in the absence of complementary evidence, the block of data described above allows proposing that ELF fields could exert its action on EGFR through prior activation of MMP-9, in a way similar to that described by other authors testing radiofrequency field effects [54]. Namely, a free radical increase mediated by an action of MF on NADH oxidase, would activate MMP-9, affecting the control of the EGFR bioavailability and activity, which in turn would further activate the ERK cascade.

On the other hand, the sulphur atom of the cysteine in the prodomain of metalloproteinases (proMMP) has been shown to inactivate the catalytic zinc-containing domain of the MMP. Activation of the enzyme, therefore, requires either proteolytic removal of the propeptide or disruption of the Zn^{2+}-cysteine bond [55, 56]. From this, and considering that the applied MF parameters of frequency and B_{DC} (DC offset = 15 μT rms) approach the resonance conditions

for the sulphur ion according to the ion parametric resonance model IPR [57- 59], a potential MF action on the sulphur atom could disrupt the Zn^{2+}-cysteine junction in the catalytic center of MMP-9, causing its activation. The scission of the EGFR ectodomain mediated by activated MMP-9 [60], could trigger the subsequent activation of EGFR. The potential implication of resonance phenomena in the MF effects on the metalloproteinase is currently investigated by assaying the action of MMP-9 inhibitors.

As summarized in the diagram of Fig. 7, taken together with our previously published data, the herein reported results reveal that the proliferative response observed in NB69 after 63 h of intermittent exposure to a weak, 50 Hz field, could be mediated by a MF-induced early activation of EGFR (at about 10 min of exposure) where a MMP-9 would be implicated (at 5 minutes of the exposure onset), which in turn would trigger transient activation of MAPK p38 (at about 15 - 30 min of exposure). Such activation would be free radical-dependent, as it can be blocked by N-acetylcysteine (NAC) [22]. Shortly thereafter, the activated EGFR would also trigger a transient activation of ERK1/2 through a free radical independent process, not blocked by NAC. The activation of both pathways p38 and ERK1/2, repeated in each of the ON cycles according to the sequence described, would be necessary for the MF to elicit the proliferative response, which would be inhibited by the blocking of either of the two pathways. On the other hand, EGFR activation by the MF would also mediate the early and transient activation of JNK. Although previous data on chemical inhibition indicate that this effect would not intervene in the proliferative response to the field, the possibility cannot be disregarded that EGFR-mediated activation of JNK could be involved in other processes, like cell survival o apoptosis, having a potential impact on proliferation.

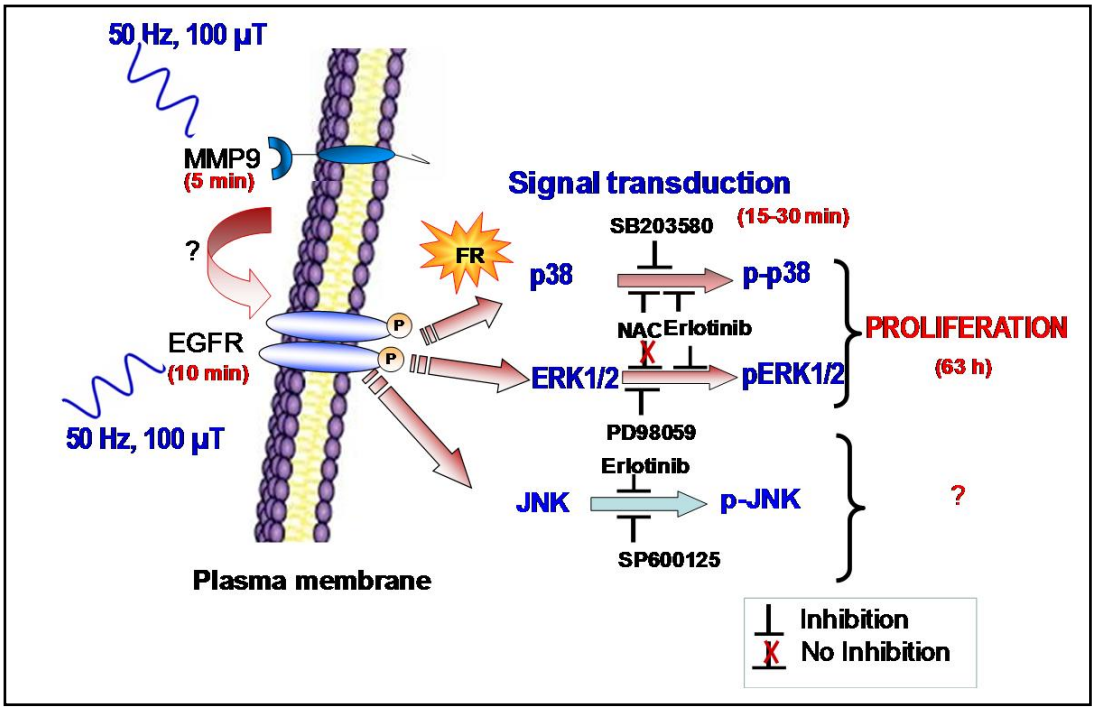

Fig. 7. Proposed early signaling mechanism triggered in NB69 cells by the 50-Hz field stimulus. The EGF receptor (EGFR) activation, potentially mediated by prior activation of MMP-9, would trigger phosphorylation of p38 and ERK1/2 pathways, which are known to be involved in the proliferative response of NB69 to the MF. EGFR would also mediate the activation of JNK by the MF, though this signaling pathway may not be involved in the proliferative response to the MF, as indicated by previously published data. The results of the tests using chemical inhibitors show that both, the p38 pathway phosphorylation, which is mediated by free radicals (FR), and the FR-independent phosphorylation of the ERK1/2 pathway, have to take place simultaneously in order that the repeated response to the intermittent field exposure leads to the proliferative effect observed at 63 h of treatment (see Discussion for detailed explanation).

Conclusion

In summary, added to previously published data, the present results allow proposing an early signaling mechanism that is triggered by intermittent exposure to weak, power frequency MF, and is potentially involved in the field-induced proliferative response of human neuroblastoma cells NB69. Said mechanism would involve the EGF membrane receptor in the activation of the MAPK signal transduction cascades p38 and ERK1/2, which would result in cell proliferation promotion. This model is fully compatible with a potential field effect on primary targets at the cell membrane level, like membrane surface charges [61-63].

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Disclosure Statement

The authors have no conflicts of interest to declare.

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