Antagonistic Effects of a 50 Hz Magnetic Field and Melatonin in the Proliferation and Differentiation of Hepatocarcinoma Cells

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Abstract

Background/Aims: Epidemiological and experimental evidence exists indicating that exposure to weak, extremely low frequency magnetic fields (ELF - MF) could affect cancer progression. It has been proposed that such hypothetical action could be mediated by MF-induced effects on the cellular response to melatonin (MEL), a potentially oncostatic neurohormone. The present study investigates the response of HepG2 cells to intermittent exposure to a 50 Hz, 10 μT MF, in the presence or absence of MEL at physiological (10 nM) or pharmacological doses (1 μM).

Methods: The Trypan blue cell exclusion test, BrdU incorporation and PCNA expression assays were carried out to assess the cellular response in terms of viability and proliferation. In addition, albumin and alpha-fetoprotein, were analyzed as specific hepatocellular differentiation markers. Results: The results indicate that the MF exerts significant cytoproliferative and dedifferentiating effects that can be prevented by 10 nM MEL. Conversely, MEL exerts cytostatic and differentiating effects on HepG2 that are abolished by simultaneous exposure to MF.

Conclusion: As a whole, these results support the hypothesis that ELF - MF and MEL exert opposite, mutually counteracting effects on cell proliferation and differentiation.

Introduction

A number of epidemiological studies have reported observations suggesting a potential relationship between the exposure to weak, extremely low frequency (ELF) environmental magnetic fields (MF) and increased cancer risk [1-3]. These observations have received partial support from in vitro studies reporting ELF MF-induced changes in the activity of...
biomolecules intervening in cell cycle regulation, cell proliferation and cell survival [4-6]. Nevertheless, due in part to the fact that the in vitro studies have generated conflicting results, it is still unclear whether and how MF exposure could promote carcinogenesis. Indeed, whereas some authors have detected no field effects on different biosystems [7-9], others, including our group, have reported significant changes in cell growth or in cancer progression in response to ELF fields or ELF-modulated RF signals [10-12]. Anyhow, on the basis of the available evidence, it has been proposed that the field-induced cellular alterations, in combination with the effects of certain risk factors and/or of physiological/environmental agents, may influence tumorigenic processes [13-16].

On the other hand, there is a large body of evidence indicating that the indoleamine hormone MEL exerts oncostatic effects on different types of animal and human tumors [17-20]. In vitro, MEL has been reported to inhibit cell growth in prostate, breast and colon cancer cells, as well as in glioma, melanoma, and hepatoma cell lines [21-27]. Also, MEL reduces invasiveness and metastasis in cancer cells [28, 29], induces apoptosis in vivo and in vitro models (review by Sanchez-Hidalgo et al. [30]) and effectively modulates junctional transfer and differentiation in a variety of cellular species [31-35]. Moreover, the administration of melatonin in conjunction with chemoradiotherapy has been associated with improved outcomes of tumor regression and survival of cancer patients [see 36 for a recent review].

It has been proposed that ELF MF exposure could affect cell homeostasis and influence cancer development through inhibition of MEL synthesis or alteration of the MEL functions at the cellular level. The hypothesis that ELF MF could promote breast cancer through suppression or alteration of the circadian pattern of MEL synthesis in humans was first proposed by Stevens in 1987 [37]. A revision of this hypothesis has been published recently [38], proposing that ELF MF-mediated disruption of circadian rhythmicity can promote the development of other cancer types, including childhood leukemia. On the other hand, the possibility that ELF-MF could interfere with the cellular response to MEL has received partial support from a number of experimental in vivo and in vitro studies [39-45]. On the basis of the above, we propose that the analysis of the in vitro responses to ELF MF and MEL, applied separately or in combined treatments, can provide information that is useful to identify basic phenomena underlying the MF interactions at the cellular level. Such information can be of pivotal interest to evaluate the potential health impact of the exposure to weak, environmental MF.

The HepG2 cell line from a human hepatocarcinoma (HCC) is a well-characterized line, suitable for cytotoxicity assessment because of the stability of its metabolic background. These cells, which retain the capability of synthesizing and secreting various liver proteins, including albumin and apolipoproteins, have shown to be responsive to weak magnetic and electric stimuli [12, 46] as well as to MEL [24, 47]. Because of this dual sensitivity, HepG2 is a particularly suitable model for our study. Besides, hepatocarcinomas are among the most common cancers worldwide and are intrinsically refractory to chemotherapy [see 48 and 49 for recent reviews]. Hence, the study of the possible effects of MF and MEL on the proliferation and differentiation of HCC cells could provide information of potential relevance in oncology.

In sum, the present work investigates the response of HepG2 cells to treatments with a 50-Hz, 10 µT MF and/or MEL at physiological or pharmacological concentrations. The cellular response was evaluated in terms of viability, proliferation, and differentiation. As a whole, the results suggest that weak, ELF MF can influence cancer-related processes in vitro, and reinforce the hypothesis that these fields could interfere with the potentially antitumoral action of MEL.

Materials and Methods

Cell culture

HepG2 human hepatocarcinoma cells (ECACC, TDI S. A., Madrid, Spain) were grown in Dulbecco’s-Eagle modified medium with 4.5 g/l glucose (DMEM; Bio-Whittaker, Verviers, Belgium), supplemented
with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL Invitrogen, Prat de Llobregat, Spain), 2 mM Glutamine and 100 Uds/ml penicillin/streptomycin (Gibco BRL, Invitrogen). The cells were kept in T-75 flasks (Sardstedt, Barcelona, Spain) inside water-jacket incubators (Forma Scientific, Thermofisher, Waltham, MA, USA) at 37 °C in a 5% CO₂ and 100% RH atmosphere, over a maximum of 25 passages. The samples were routinely tested for mycoplasma and other bacterial contamination, through standard microbiological procedures using SP4 agar or broth media, ureaplasma differential agar medium, arginine broth and urea broth.

Melatonin (N-acetyl-5-methoxy-tryptamine, Sigma-Aldrich, Madrid, Spain) was dissolved in 0.5 ml of 95% ethanol, at a 10⁻² M concentration, according the procedure established by Blackman et al. [43]. Immediately before each experimental run, this stock solution was diluted in culture medium to obtain MEL concentrations ranging 0.01 nM to 1 µM, and the corresponding concentrations of vehicle, within the 1:10⁻⁵ to 1:10⁻¹⁰ v/v range. In order to discard potential artifacts, a pilot test with two control groups, with and without vehicle, was conducted using the highest vehicle concentration to be assayed in the experiments. Three repeats, each with three dishes per experimental condition, were carried out. No significant differences on cell growth were detected when the samples treated with vehicle were compared to the untreated ones (data not shown).

Exposure System

The exposure set-up has been described before by Trillo et al. [12]. Briefly, the system consists of a function generator (Newtronics Model 200MSTPC, Madrid, Spain) and an ammeter (Hewlett Packard, model 974A, Loveland, CO, USA), connected in series to two identical pairs of coils. Each coil was made of 1000 turns of enameled copper wire. The coil pairs, set in a Helmholtz configuration, were coaxially oriented to produce a vertically polarized magnetic field. The magnetic field parameters were routinely checked with magnetometers (EFA-3, Model BN 2245/90.20, Wandel & Goltermann S.A Eningen, Germany and EMDEX II, Enertech Consultants, Campbell, CA, USA). Each of two coil pairs was placed inside each of two identical, magnetically shielded chambers (co-netic metal; Amuneal Corp., Philadelphia, PA, USA) located in two identical CO₂ incubators (Forma Scientific) with a 5% CO₂ and 100% humidity atmosphere at 37 °C (Fig. 1). The ambient DC and AC (50 Hz) fields inside the shielded chambers were 0.04 ± 0.03 μT rms and 0.05 ± 0.04 μT rms, respectively. The MF exposure parameters were chosen on the basis of previous knowledge on cellular response to 50 Hz MF [12, 16, 50]. For MF treatment, 60 mm Petri dishes (Nunc, LabClinics, Barcelona, Spain) containing the cell cultures were placed within the uniform MF space created by the paired Helmholtz coils. In each experimental run only one of the two coil sets was energized for MF exposure. The samples in the incubator containing the non-energized coils were considered sham-exposed controls. Both incubators were used alternatively for MF-exposure or sham-exposure, in a random sequence.

Cell viability and proliferation assays

Cell viability and proliferation were determined through Trypan blue (Sigma-Aldrich) exclusion and hemocytometer counting. Briefly, the cultures were seeded at a density of 9 x10⁴ cells/ml in 60 mm Petri dishes. Once the cells attached to the dish surface (4 hours post-plating), they were incubated and treated as described in Fig. 2, part 1 and part 2. At the end of the experiments, the cells were harvested and collected in 1 ml of medium. Cells were stained with 200 µl of 0.04% Trypan blue in 0.5 ml Dulbecco’s PBS (Gibco BRL, Invitrogen) and evaluated under a light microscope.
BrdU incorporation assay
Following the general procedure (Fig. 2, part 2) the samples were seeded on pairs of circular, 12 mm diameter coverslips (Hirschmann Laboratories, Eberstadt, Germany) placed inside the Petri dishes, and treated with MF and/or MEL (10 nM or 1 μM). At day 4 after seeding 5 μM BrdU (Sigma-Aldrich) was added to the samples. The amount of BrdU incorporated by the cells was quantified after an additional period of 18 hours of exposure and/or incubation, in the presence or the absence of MEL. For indirect immunofluorescence analysis the cultures were incubated in the presence of a primary monoclonal antibody anti-BrdU (clone Bu20a, Dako, Barcelona, Spain) and a secondary anti-mouse antibody conjugated with fluorescein (Amersham, Buckinghamshire, UK). The nuclei were counterstained with Hoechst 33342 (Bisbenzimide, Sigma-Aldrich). Background controls without BrdU were included in the study. The samples were studied through photomicroscopy (Nikon Eclipse TE300; Melville, NY, USA) and Computer-Assisted Image-Analysis (AnalySIS: Soft-Imaging Systems GmbH, Münster, Germany).

In each of three experimental runs, 2 pairs of coverslips corresponding to 2 Petri dishes were analyzed per experimental condition: Control, MEL (10 nM and 1 μM), MF, MEL + MF. Twenty random microscope-fields per coverslip were evaluated. In each microscope-field the total nuclei (Hoechst 33342 fluorescent dye) and the percent of BrdU positive cells were recorded. A total of about 4500-5000 cells per experimental group were evaluated in each experimental run.

Immunocytochemical analysis
The cells were seeded on 12 mm diameter coverslips placed inside Petri dishes and treated for five days (Fig. 2, part 2). PCNA expression was estimated through indirect immunofluorescence. The cells on coverslips were incubated with a monoclonal antibody anti-PCNA (FL-261; Santa Cruz Biotechnology, Quimigen S.L., Madrid, Spain) and with an Alexa Green secondary antibody (Molecular Probes, Invitrogen, Prat de Llobregat, Spain). Hoechst 33342 was added to the mounting medium as a counterstain for nuclei. In each of three experimental runs, 2 pairs of coverslips corresponding to 2 Petri dishes were analyzed per experimental condition (Control; 10 nM MEL, MF, MEL + MF). In each coverslip ten microscope fields were selected at random and analyzed. A total of about 2500-3000 cells per treatment were examined in each experimental run.

Western blot assay
The cellular expression of alpha-fetoprotein (AFP) was analyzed by Western-blot in samples treated with MF and/or MEL (10 nM or 1 μM) for five days, following the general procedure (Fig. 2, part 2). The proteins were extracted in hypotonic lysis buffer. A total of 50 μg protein per sample were separated by SDS-PAGE and transferred to nitrocellulose membranes with a semi dry system (BioRad, Munich, Germany). The membranes were immunostained with a primary antibody against AFP. Equal loading of protein was demonstrated by probing the membranes with a monoclonal antibody anti β-actin (clone AC-15; Sigma-Aldrich). The blots were revealed by ECL-chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and the bands obtained were evaluated by densitometry (PD Quantitation One 4.5.2 software, BioRad). In each of eight experimental replicates, two samples were studied per experimental condition.

Nephelometry
At the end of the fifth day of treatment (Fig. 2, part 2) with MF and/or MEL (10 nM or 1 μM), the cell number was quantified and 3 ml of the medium were collected from each dish and frozen at -80 °C. In each experimental run an additional sample of 3 ml of fresh medium was collected and frozen for determination of basal albumin (ALB) concentration. For nephelometric analysis, the samples were lyophilized, resuspended in water and processed in a Nephelometer BN II (Dade Behring, Barcelona, Spain). The albumin antiserum, the internal control, and the buffer for the reaction were purchased from Dade Behring, and used following the manufacturer’s recommendations. An additional, external control was also used (BioRad). The resulting data were normalized for volume and cell number. In each of eleven experimental replicates, two samples were studied per experimental condition.

Dose response to MEL: cell viability and proliferation assay
The standard procedure summarized in Fig. 2, part 1 was applied. In order to determine the optimal concentration of melatonin for induction of an antiproliferative response, five concentrations of MEL were assayed: 0.01 nM, 0.2 nM, 0.4 nM, 10 nM and 1 μM. These concentrations cover the range at which MEL
has been reported to affect proliferation-related processes in vitro [21, 39, 43, 51-53]. Untreated samples were also included for assessment of HepG2 proliferation rate in control conditions. At hour four post-plating each dish received the corresponding dose of MEL. After an additional interval of 72 h the media were discarded and replaced with fresh medium, preheated at 37 °C and supplemented with fresh MEL. This standard procedure, which took a maximum 5 minutes, is known not to influence significantly the cell growth rate or the cellular response to the imposed treatments. The cellular response was analyzed two days later, at day 5 post-plating, using the Trypan blue cell exclusion test. In each of a minimum of six experimental repeats per dose of MEL, 3 to 5 dishes were analyzed.

**Cell response to MF in the presence or absence of melatonin: viability and proliferation assay**

In a first series of experiments, the proliferative response to combined treatment with MF and 10 nM MEL was analyzed at the end of days 4, 5 and 7 post-plating. In each of fifteen experimental replicates, a total of 12 - 20 Petri dishes (3 - 5 dishes per experimental condition) were exposed to one of the following treatment combinations: MEL-/MF-; MEL+/MF-, MEL-/MF+ or MEL+/ MF+. As shown in Fig. 2, part 2, after allowing for cell attachment to the dish surface, the samples were distributed in two groups. One group received an initial, physiological dose of 10 nM MEL; the other group received the corresponding dose of vehicle (ethanol at a final concentration of 10⁻⁷ % v/v). At day 3 post-plating, the media were renewed and a second dose of 10 nM MEL or vehicle was administered to the treated samples. Each of the two groups, treated with MEL or untreated, were distributed in sham-exposed and MF-exposed samples. The MF-exposed samples were treated intermittently, 3 h On / 3 h Off cycles, with a 50 Hz, 10 µT MF during 24, 42 or 90 hours. At the end of the MF and/or MEL treatment the cells were studied for viability and proliferation using the Trypan blue cell exclusion test.

In two additional series of experiments the proliferative response to combined treatment with MF and MEL (10 nM or 1 µM) was analyzed at the end of the day 5, through Trypan blue cell exclusion and BrdU incorporation. In each of thirteen experimental replicates, a total of 12 - 20 Petri dishes (3 - 5 dishes per experimental condition) were submitted to one of the following treatment combinations: untreated controls, MEL (10 nM or 1 µM), MF or MEL + MF.

**Data analysis**

All procedures were conducted in the blind for comparative analyses of the MF- or MEL-treated vs. sham-treated samples. Data were normalized and expressed as means ± standard error (SEM) of at least three independent replicates, using GraphPad Prism software (Graphpad Software, Inc., San Diego, CA, USA). The normalized data were tested for gaussian distribution by the Kolmogorov-Smirnov test. The ANOVA test followed by two tailed Student’s t-test was applied for analysis of statistical differences between treatments. Differences p<0.05 were considered significant statistically.
Results

Dose–response to MEL: Viability and Proliferation

Although the physiological levels of plasma MEL in mammals are in the nanomolar range, MEL concentrations several orders of magnitude higher have been detected in some tissues, like the gastrointestinal tract [54, 55]. Among the MEL doses tested in the present study, those between 0.01 nM and 10 nM are within the physiological range [33], whereas 1 μM can be considered a pharmacologic dose. None of these doses affected significantly the viability rate of the HepG2 cultures (about 98% in all cases, data not shown). As shown in Fig. 3, 0.01 nM MEL induced a slight but statistically significant increase in the number of cells (6.4% p<0.05) at day 5 of treatment. In contrast, higher concentrations of MEL: 0.4 nM, 10 nM and 1 μM, induced modest but significant decreases in cell number: 6.0% (p<0.01) 6.5% (p<0.001) and 5.6% (p<0.001) below controls, respectively. Since 10 nM was the physiological dose that induced the most consistent anti-proliferative effect, this concentration was selected, along with that of 1 μM, to test and compare the MF effects in the presence of physiological or pharmacological doses of MEL.

Viability and Proliferation under treatment with MF and/or MEL at days 4, 5 and 7 post-plating

None of the combined or separate treatments affected cell viability (≈ 98% in all cases). However, both agents, MEL and MF, induced changes in the proliferation rate of HepG2. As shown in Fig. 4, at day 4 post-plating and in the absence of MF exposure, 10 nM MEL induced a transitory increase (5.2%; p<0.05) in the number of cells relative to controls. At that time and in the absence of MEL, the MF also increased cell number significantly (15.3%; p<0.001). Such MF effect was blocked by the presence of MEL, the cultures submitted to combined treatments reached a cell number significantly lower than that with MF only (p<0.05).

Twenty-four hours later, at day 5 post-plating, 10 nM MEL induced a significant decrease in cell number (8.0% below controls; p<0.001, Fig. 4), which confirms and reinforces the results of the dose-response test (Fig. 3). The MF-induced proliferative effect observed at day 4 remained at day 5 (9.6 % vs. controls; p<0.001). However, in the combined treatment, with MF + MEL, the number of cells was equivalent to that in controls, and significantly different from that reached after treatment with MF only (p<0.01) or MEL only (p<0.05). Thus, at day 5 the presence of MEL in the medium prevented the cultures from responding to the proliferative stimulus of the field, similar to the results at day 4. In turn, the MEL-induced antiproliferative response observed at day 5 was abolished by simultaneous exposure to the MF.

On day 7 post-plating, the antiproliferative effect of MEL observed at day 5 seemed to persist, though the differences with respect to controls did not reach statistical significance. The MF, either alone or in combination with MEL, induced significant increases in the cell number: 25.3% (p<0.01) and 19.7% (p<0.05) over controls, respectively. Thus, in contrast to that observed at day 5, two days later the presence of MEL in the medium did not antagonize the field effect. Since this could be due to depletion of MEL in the culture medium, it was decided that for the rest of the study the cellular response would be analyzed at day 5 post-plating.

Comparative analysis of DNA synthesis and proliferation after treatment with MF and/or MEL

As shown in Fig. 5A, MEL doses of 10 nM or 1 μM MEL induced reduction in DNA synthesis at day 5 post-plating as measured by BrdU incorporation (20.3% and 33.2% below controls, respectively; p<0.05). When administered alone, MF induced significant increase in BrdU incorporation (41.7% over the corresponding controls; p<0.05). However, when the field exposure took place in the presence of MEL, the BrdU incorporation was significantly reduced with respect to untreated controls, at the dose of 1 μM (37% below controls; p<0.05), and with respect to MF-exposed samples, at 10 nM and 1 μM. Thus, MEL can block and revert the MF-induced increase in BrdU incorporation.
In a matched set of experiments, the proliferative response to the same treatments was analyzed (Fig. 5B) and compared to that of BrdU incorporation. MEL at 10 nM and 1 µM induced significant, equivalent decreases in cell number (5.5% and 5.7%, below controls; p<0.001 and p<0.01, respectively). On the other hand, MF exposure significantly increased cell number (8.1% over controls; p<0.001). As for combined treatment with MEL + MF, the obtained cell count was similar to that of controls, indicating that MEL can prevent the MF-induced proliferative response. Further analyses should be conducted on day 6 post-plating or later, in order to determine whether the MEL-induced reversion of the stimulated DNA synthesis in MF-exposed samples, observed at day 5, could subsequently lead to an equivalent reversion of the MF cytoproliferative effect.
PCNA expression was analyzed at day 5, after MF exposure and/or treatment with 10 nM MEL. PCNA is a protein auxiliary of DNA polymerase-δ, synthesized in late G1 and S phases of the cell cycle. PCNA expression correlates with the proliferative status of the cell and is involved in DNA replication and repair [56]. In samples treated with MF, alone or in combination with MEL, the PCNA expression was significantly increased (Fig. 6), which could be indicative of a MF-induced increase in the proportion of cells in the S phase, or of an arrest of the cell cycle in the S phase. This duality can be addressed through data analysis of BrdU incorporation and PCNA: in the absence of MEL, the MF seems to stimulate the kinetics of the cell cycle, increasing DNA synthesis and PCNA expression. In the combined treatment, the MEL-induced antiproliferative effect could be mediated by a blocking of the DNA synthesis, leading to the observed decrease in BrdU incorporation and increase in PCNA expression.

**Assessment of MF and MEL effects on cell differentiation: Albumin release and alpha-fetoprotein expression**

As shown in Fig. 7, MEL concentrations of 10 nM and 1 μM significantly increased the levels of albumin released to the medium by the cells (35.6% and 7.9% over controls; p < 0.001 and p < 0.001, respectively), with some of the samples treated with 10 nM MEL being particularly responsive to the hormonal stimulus. By contrast, MF exposure alone significantly diminished ALB levels in the medium (20.6% below controls; p<0.05), and when applied to hormone-treated samples, the magnetic stimulus fully reverted the 1 µM MEL-induced release of ALB, or partially antagonized the effect of 10 nM MEL.

Alpha-fetoprotein expression was also examined under the same treatment conditions. Fig. 8A and 8B show that MEL at 10 nM or 1 μM significantly decreased AFP expression (30.8% and 40.2% below untreated controls, respectively; p<0.001), whereas MF alone increased significantly the expression of this protein (14.3%; p<0.05). In the combined treatment, the exposure to the field antagonized the decrease in AFP expression induced by 10 nM MEL, reaching expression levels similar to those in controls. When administered in the presence of 1 μM MEL, the MF promoted AFP expression, reaching levels significantly higher than those in untreated controls (30.9%, p < 0.01) and similar to those in samples exposed to MF only. Thus, again, the physiological dose of 10 nM MEL was more consistent than the pharmacological concentration in antagonizing the MF-induced cellular response.
Discussion

The above described results show that intermittent exposure to a 50 Hz, 10 μT MF can induce cell proliferation and dedifferentiation in the human hepatocarcinoma line HepG2, without affecting the viability of the cultures. This reinforces prior observations on in vitro effects of power-frequency fields, recently reported by our group [12, 16]. The proliferative effect was accompanied with increased incorporation of BrdU at 24 and 42 hours of exposure, together with increased expression of PCNA at 42 h. These results are indicative that the field exposure can upregulate HepG2 proliferation through stimulation of DNA synthesis. Such stimulation seems to occur relatively early after the exposure onset, since the subsequent proliferative effect was already detected at 24 h of treatment. As for differentiation, the MF exposure decreased the release of ALB, a protein that is specific of adult hepatocytes, and increased expression of protein AFP, typically found in poorly differentiated hepatocytes. These effects are indicative that MF can cause de-differentiation in HepG2 cells. A similar modulation of ALB release and AFP expression has been described in HepG2 cells grown under conditions that promote the selection of specific cellular subtypes [57]. Considering this, in the present study the proliferative response elicited by the field might lead to changes in the proportions of the different cellular phenotypes in the cultures, favouring the expansion of one or more specific cell subtypes. Moreover, the stimulation of AFP expression by MF may involve an autocrine stimulation of cytoproliferation, as described by Li et al. [58] in hepatoma cells, enhancing the field-induced proliferative effect. Thus, the present data indicate that besides exerting a proliferative action, the MF could promote cell dedifferentiation, which might result in selection of actively proliferating phenotypes.

Direct increase of proliferation and DNA synthesis, as well as other effects related to stimulation of cell division and alteration of cell differentiation, have been reported in different cellular models exposed to ELF fields at high magnetic flux densities. For instance, significantly increased growth rate and decreased proinflammatory chemokine production have been described on human keratinocytes (HaCaT) exposed to 50 Hz, 1 mT MF [59]. Other authors have described alterations in the kinetics of the cell cycle and/or in its regulatory proteins, under exposure to ELF fields [4, 60, 61]. Also, Ke et al. [62] reported EGF-receptor clustering and Ras activation in Chinese hamster lung fibroblasts exposed to 400 μT MF. More recently, we have reported that a 50 Hz, 100 μT MF can stimulate proliferation and activation of ERK1/2 in the human neuroblastoma line NB69 [50]. As a whole, these and other in vitro effects are largely dependent on the cell type and on the field parameters. For instance,
Sul et al. [61] reported that 3 h/day exposure to a 60 Hz, 2 mT MF stimulates proliferation in the lymphoblastic cell line RPMI 7666, but inhibits proliferation in muscle cells T/G HA-VSCM. By contrast, pulse wave, 16 - 80 Hz MF at 1.55 mT did not affect significantly proliferation in HepG2 [63]. This specificity in the cellular response could explain in part the inhomogeneity in results reported by different authors using different exposure conditions or different \textit{in vitro} models, as proposed by Ross [64] or Zhang et al. [65].

Moreover, the experimental evidence suggests that different molecular mechanisms can be involved in the action of ELF MF on cell proliferation. Some of these mechanisms would be responsible for MF-induced alterations in Ca\textsuperscript{2+} flux [66, 67]. Also, changes in the functionality of receptors have been observed, the Ras-Raf-ERK transduction cascade being a potential pivotal target for ELF fields [62, 68]. Increased activation of mitogen-activated kinase and extracellular-regulated kinase (MAPK/ERK) in response to 50/60 Hz MF have also been reported in NB69 [50], HL60 and MCF-7 cells [69]. It has been also proposed that oxidative stress disturbances due to MF-induced changes in the redox status of the cell could be one of the causes underlying the wide variety of cellular alterations reported in the literature [5, 70].

Thus, the results of the present work are coherent with the current evidence on the cytoproliferative response to ELF MF. However, most studies conducted so far have assayed magnetic flux densities above the reference levels of 100 µT or 500 µT proposed by the International Commission on Non-Ionizing Radiation Protection (ICNIRP) [71], for protection of the general public or of the workers, respectively, against potential harmful effects of short term exposures to 50 Hz MF. In contrast to that, the present study describes the cellular response to 10 μT, a significantly lower flux density that can be found in specific residential and occupational environments [72].

As for the effects of MEL on cytoproliferation, the present results indicate that this neurohormone exerts in HepG2 a biphasic, non-linear effect, depending on its concentration and on the duration of the treatment. When administered at a low, physiological concentration during less than 5 days, MEL induced significant increase in cell number. An opposite, antiproliferative response was obtained after 5 or more days of treatment with doses of MEL ≥0.04 nM. This is coherent with the results of a number of studies reporting biphasic effects of melatonin on different cellular processes, including growth, viability and apoptosis, depending on the cell type and on the MEL concentration [73-75]. This type of dual patterns has been repeatedly described in the \textit{in vitro} response to agents that exhibit antitumoral activity [76, 77].

In the present study, the antiproliferative effect of MEL at doses ≥10 nM was associated to a significant decrease in BrdU incorporation that did not involve changes in PCNA expression. This is indicative that MEL can partially block DNA synthesis without preventing the cells from entering in S phase of the cycle. Thus, the observed cytostatic effect of MEL could be linked to a partial blockade of the DNA synthesis. Experimental evidence exists indicating that high doses of MEL, in the mM range, can exert cytocidal effects in HepG2 [73, 78]. In our study, the decrease in cell number induced by 1.0 µM MEL would not be attributable to cytotoxicity, since the effect was not accompanied by changes in cell viability. This adds to the block of evidence that, depending of the dose, MEL can specifically trigger different cellular processes. Indeed, the effects of high, pharmacological doses of MEL have been associated primarily with non-specific properties of the hormone, such as free radical scavenging [79]. In turn, the effects of low, physiological doses, would preferentially intervene in specific actions of MEL, based on interactions with membrane receptors or on modulation of signalling cascades.

Our results also show that the antiproliferative response to physiological or pharmacological doses of MEL is accompanied by increased ALB release and decreased AFP expression, which are indicative of cell differentiation promotion. Other pro-differentiative effects of MEL have been reported in other cell lines [25, 33-35, 80]. Hence, the present results represent an additional support to the evidence indicating that MEL could exert a function in controlling carcinogenic processes.
Concerning the response to the combined treatment with MF and MEL, the antiproliferative effects exerted by 10 nM or 1 μM MEL at day 5 of treatment were abolished by a 42-h, intermittent exposure to the 50 Hz, 10 μT MF. In turn, the proliferative action of the field was prevented by the presence of MEL in the medium. Furthermore, 10 nM and 1 μM MEL partially blocked DNA synthesis, both in the absence and in the presence of MF stimulus, as estimated through BrdU incorporation. In particular, the fraction of BrdU-positive cells in the MF+MEL-treated samples was significantly reduced with respect to that in the group exposed to MF only. Antagonistic actions between MEL and ELF MF has been reported before in breast cancer cells [43, 81, 82], in which the phenomenon was linked to a field-mediated uncoupling of the adenylate cyclase pathway regulated by melatonin receptors [44, 45]. The possibility that such receptors might also be involved in the herein described MEL-MF interactions in HepG2 remains to be investigated.

Regarding differentiation under combined treatment, antagonistic interactions between ELF MF and differentiation promoters like NGF, MEL or retinoids, have been reported in different cell types [12, 40, 83, 84]. The present results also show that the MF antagonizes the effects on AFP expression and ALB release, exerted by the assayed doses of MEL. In turn, the presence of a physiological dose of 10 nM MEL prevented the dedifferentiating action of the MF, whereas a higher, pharmacological dose of 1 μM was ineffective in that respect. Again, this adds to the block of evidence indicative that certain cellular effects of MEL are mediated by mechanisms triggered specifically by physiological doses of the hormone [21, 39, 75]. The general evidence in vitro indicates that the effective range of MEL at physiological doses appears to be narrower in normal cells than in transformed lines [85]. In this respect, the present results show that HepG2 displays cytostatic and differentiating responses at a MEL dose of 10 nM, whereas in normal cells such type of effects have been reported to occur at lower concentrations [32].

In sum the overall results show that 24 h, 42 h or 90 h of intermittent exposure to a 10 μT, 50 Hz, sine wave, linearly polarized MF promotes cell proliferation in the HepG2 cell line. Such an effect was prevented by supplementing the culture medium with physiological or a pharmacological doses of MEL. Conversely, MEL exerts an antiproliferative effect in HepG2 that is blocked by the MF stimulus. These results extend the body of experimental evidence on the antagonistic action of MF on MEL-mediated cytostatic effects [43, 44, 81, 82]. Moreover, our results are indicative that MF can elicit dedifferentiating responses and neutralize MEL-induced stimulation of cell differentiation. In turn, physiological, but not pharmacological doses of MEL can prevent the HepG2 cells from responding to the dedifferentiating action of the MF. As a whole, the present results provide new evidence on the potential proliferative action of weak, power frequency MF and indicate that MEL and MF elicit antagonistic responses in HepG2 cells. This is coherent with the hypothesis that exposure to weak, ELF magnetic fields can stimulate cancer progression by enhancing growth or dedifferentiation of proliferating cancerous cells and/or by inducing clonal expansion of specific cell types. The observed antagonistic action of MEL also supports the hypothesis that individuals with low levels of plasma MEL could be particularly vulnerable to the potential adverse effects of MF, and that administration of supplementary MEL might help those individuals to avoid such effects. Besides, the cytostatic effects exerted by MEL on HepG2 are consistent with clinical data showing that MEL can be an effective adjuvant in the treatment of patients with hepatocellular carcinoma [86, 87].

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