

# Acute and chronic effects of exposure to a 1-mT magnetic field on the cytoskeleton, stress proteins, and proliferation of astroglial cells in culture<sup>☆</sup>

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## Abstract

This paper reports the effects of exposure to static, sinusoidal (50 Hz), and combined static/sinusoidal magnetic fields on cultured astroglial cells. Confluent primary cultures of astroglial cells were exposed to a 1-mT sinusoidal, static, or combined magnetic field for 1 h. In another experiment, cells were exposed to the combined magnetic field for 1, 2, and 4 h. The hsp25, hsp60, hsp70, actin, and glial fibrillary acidic protein contents of the astroglial cells were determined by immunoblotting 24 h after exposure. No significant differences were seen between control and exposed cells with respect to their contents of these proteins, neither were any changes in cell morphology observed. In a third experiment to determine the effect of a chronic (11-day) exposure to a combined 1-mT static/sinusoidal magnetic field on the proliferation of cultured astroglial cells, no significant differences were seen between control, sham-exposed, or exposed cells. These results suggest that exposure to 1-mT sinusoidal, static, or combined magnetic fields has no significant effects on the stress, cytoskeletal protein levels in, or proliferation of cultured astroglial cells.

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## 1. Introduction

All living organisms, especially humans, are continuously exposed to electric and/or magnetic fields (EMF, MF). It now seems clear that exposure can induce biological changes, although the precise effects provoked are not well known. It is usually argued that the controversial or inconsistent results obtained so far are mainly due to the different experimental protocols that have been used. The mechanisms by which EMFs/MFs

interact with biological systems are even poorly understood (Goodman and Blank, 2002). Nevertheless, it seems that MFs may be able to induce a wide variety of effects (see reviews of Goodman et al., 1995; Hong 1995), and much attention has been paid to their effects on stress proteins (Goodman and Blank, 1998; Pipkin et al., 1999; Miyakawa et al., 2001). The mutagenic (carcinogenic) effect of MFs has also been widely studied in man and other mammals. However, no clear relationships have been demonstrated so far (McCann et al., 1998; Ansari and Hei, 2000; Loberg et al., 2000). Epidemiological evidence suggesting that exposure to EMFs may cause cancer is weak; nevertheless, the possibility that exposure is a risk factor has not yet been discarded (McCann et al., 1998; Kheifets, 2001).

Astroglial cells provide an excellent biological model to study the effect of MFs on cytoskeletal components

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and stress proteins. These are important elements in cell physiology, and a close interrelationship between them has been demonstrated (Williams and Nelsen, 1997). Besides containing microtubules and microfilaments (actin filaments), astrocytes have a very well-developed intermediate filament system. Glial fibrillary acidic protein (GFAP) is the main component of the latter in mature astrocytes (Eng, 1985), and this can be used not only as a good marker of astroglial cells but also as a useful indicator of neurotoxicity (O'Callaghan, 1991). It is well known that changes in astroglial physiology are usually reflected as changes in expression, content, and/or the degree of polymerization of GFAP. Stress proteins are expressed by glial cells (Brown, 1990), and astrocytes show particularly vigorous production (Nishimura et al., 1991). The stress protein family has received a great deal of attention in studies on the effects of MF exposure (Goodman and Blank, 1998), probably because MFs can be used to induce cytoprotective reactions (Di Carlo et al., 1998, 1999; Carmody et al., 2000).

The idea that glial cells participate in the reactions of neural tissue to MFs is not new. In fact, the primacy of glial cells in such reactions was suggested some years ago (see review of Zhadin, 2001). In an extensive occupational study (Theriault et al., 1994), an increased risk of astrocytoma was found after exposure to MFs. The possible central role of glial cells in these responses, plus the ease with which astrocytes can be identified (via their GFAP expression), renders them especially useful as a model for analyzing the biological effects of MFs. The aim of this study is to analyze the effect on cultured astroglial cells of acute MF exposure (1, 2, and 4 h) in terms of the changes in the expression of two cytoskeletal proteins (actin and GFAP) and three stress proteins (hsp25, hsp60, and hsp70). The MFs used were a 50-Hz sinusoidal (alternating current, AC) field, a static (direct current, DC) field, and a combined MF (AC/DC), all with a magnetic flux density of 1 mT. The effect on the proliferation of astrocytes of chronic exposure (11 days) to a 1-mT AC/DC MF was also determined.

## 2. Materials and methods

### 2.1. Astroglial cell culture

Primary cultures of astroglial cells were obtained from the cerebral hemispheres of PO-P1 Wistar rats. After removal of the meninges, cells were mechanically dissociated using a narrowed Pasteur pipette and two nylon filters of 135 and 20  $\mu\text{m}$ . The cells were cultured in 25-cm<sup>2</sup> flasks containing DMEM (GIBCO, Grand Island, NY, USA) supplemented with 12% fetal bovine serum (GIBCO), 100 U/mL penicillin and streptomycin

(Sigma Chemical Co., St. Louis, MO, USA) and 2.5  $\mu\text{g}/\text{mL}$  fungizone (GIBCO), at 37 °C and a 5% CO<sub>2</sub> atmosphere (Heraeus 6000 incubator, Germany). The medium was changed every 3–4 days. Cultures were confluent after 20 days. This procedure yielded greater than 95% astrocytes as determined by GFAP immunohistochemistry. Except the experiment on the effect of MFs on astroglial proliferation, all work was performed with confluent cells.

### 2.2. MF production

MFs were generated by a pair of coils ( $N = 250$ ,  $L = 2.2$  mH; Leybold, Germany) placed in the incubator but connected to a sinusoidal and/or static power supply located outside (Fig. 1). MF intensity was controlled by adjusting the coil current and measured by means of a Hall probe (Leybold–Heraeus, Germany) placed between the two coils. The MF obtained was considered uniform for the entire culture surface since variations of less than 5% were detected between the middle and the edges of the culture flask. MF intensity data are given as rms. The static MFs in the incubator (other than the geomagnetic field) had an intensity of  $<0.6 \mu\text{T}$  (measured by a Teslameter Model 4080, F.W. Bell, USA).

### 2.3. Experimental schedules

Primary astroglial confluent cultures were exposed to 1 mT (rms), since this is an MF flux intensity to which humans can be exposed in some occupational activities. Control cultures were placed under the same conditions (time, location, and incubator) as exposed cultures, with the exception that the coils were not activated. All experiments were replicated three times and flasks were always sequentially exposed.

#### 2.3.1. Effects of MFs and the presence of serum

Twelve flasks were divided into four groups. Two groups (one with and the other without serum) were exposed to a 1-mT DC MF for 1 h, and the remaining

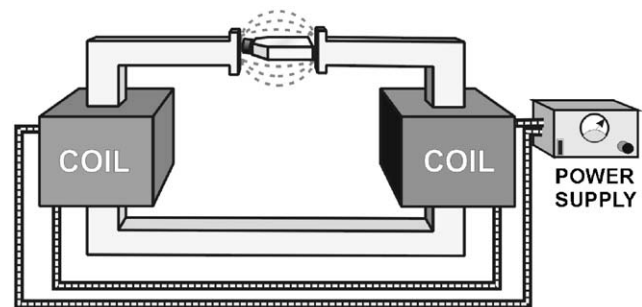


Fig. 1. Schematic drawing of the exposure system; the power supply was located outside the CO<sub>2</sub> incubator.

two (also with and without serum) were sham-exposed. Cells were collected 24 h after MF exposure.

### 2.3.2. Effects of AC, DC, and AC/DC MFs

Twelve flasks were divided into four groups: three were exposed to MFs while the fourth was sham-exposed. All were deprived of serum 24 h before exposure. The first group was exposed to a 1-mT AC (50 Hz) MF, the second to a 1-mT DC MF, and the third to a 1-mT AC/DC MF. All were serum deprived 24 h before exposure and collected after 24 h.

### 2.3.3. Time-dependent AC/DC MF effect

Twelve flasks were divided into four groups: three were exposed to a 1-mT AC/DC static MF (1, 2, and 4 h, respectively), while the fourth was sham-exposed. All were serum deprived 24 h before exposure and collected 24 h later.

### 2.3.4. Effect of AC/DC MF effect on astroglial cell proliferation

Two millions cells were plated in two 75-cm<sup>2</sup> flasks. Twenty-four hours later, a 16-cm<sup>2</sup> area was continuously exposed to a 1-mT AC/DC MF perpendicular to the flask base (Fig. 2A). In this same flask an area of equal dimensions was used as the sham-exposure area, and in a second flask a further, equally sized area was used as a control (Fig. 2B). Twelve different points were selected on each of these surfaces and an optical microscope ( $\times 20$  objective lens) was used to analyze the effects of MF exposure. A record was made of whether the  $\times 20$  objective visual field was/was not totally covered by astroglial cells after 11 days of MF exposure. The experiment was repeated three times. Data are expressed as means  $\pm$  SEM ( $n = 3$ , and 12 points per area).

## 2.4. Protein isolation

A 0.25% trypsin solution in PBS was used to detach cells from the flask. Trypsin was inactivated by adding serum, and the suspension was centrifuged (200g) and washed twice in PBS. The supernatant was discarded and the pellet was resuspended in 0.3 mL distilled water. The suspension was then sonicated (Microson XL, Mixonix) for 20 s with short pulses at 4°C before being centrifuged for 12 min at 20,000g. The pellet was discarded and the supernatant aliquoted and stored to  $-70^{\circ}\text{C}$ . A sample was used to determine protein concentration by means of a Bradford microassay procedure (Bio-Rad protein assay, Bio-Rad Laboratories GmbH, München, Germany).

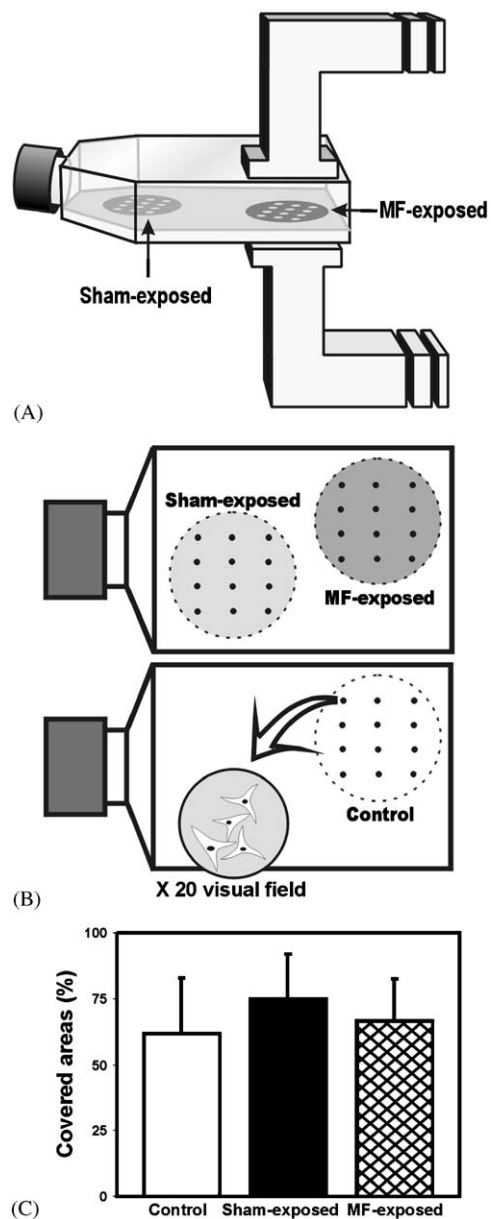


Fig. 2. (A and B) Diagrams of the experimental procedure followed in the study of the effect of chronic (11-day) static and sinusoidal 1-mT MF exposure. (C) Percentages of areas totally covered by astroglial cells after 11 days of MF exposure. Bars represent means  $\pm$  SEM;  $n = 3$  (12 points per area).

## 2.5. Western blots

Samples were prepared for electrophoresis on polyacrylamide slab gels with the addition of sample buffer to get 0.06 M Tris, 5%  $\beta$ -mercaptoethanol, 2% SDS, 10% glycerol, pH 6.8. The amount of protein per sample was obtained from the linear ranges of standard curves; 10 (hsp25), 30 (hsp60), and 15  $\mu\text{g}$  (hsp70, actin, and GFAP). The stacking gels contained 4%; the separating gels contained 12% acrylamide. After electrophoresis, proteins were transferred to immobilon-P

sheets (Millipore Corp., Bedford, MA, USA). These were washed in Tris buffer (0.05 M, pH 7.6) for 5 min and incubated with agitation for 30 min at room temperature in Tris buffer with 5% (w/v) nonfat powdered milk (blotto buffer). Without washing, the sheets were incubated overnight at 4 °C with primary antisera (Table 1). After three 5-min washes with agitation (once in blotto buffer and twice in Tris buffer), the sheets were then incubated with the appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature. They were then rinsed once again in blotto buffer and twice in Tris buffer. The peroxidase reaction was carried out in a solution of 0.05 M Tris buffer containing 0.1% 3,3-diaminobenzidine (Sigma Chemical Co.) and 0.05% H<sub>2</sub>O<sub>2</sub>. Control sheets were used to test antibody specificity; one with plasma proteins and the other without primary antiserum. These control sheets showed no immunoreactive pro-

duct. A blind analysis was followed in the videodensitometric procedure (Scion Image, Scion Corp., Frederick, MD, USA) used for immunoblot immunoreactivity quantification. Results are expressed as mean optical density (the sum of the gray value of all pixels divided by the number of the pixels). Data are expressed as the means ± SEM (*n* = 3, and three flasks per group).

2.6. Statistical analysis

The Student *t* test was used to compare the protein levels of control and exposed cells. Significance was set at *P* < 0.05.

3. Results

Fig. 3A shows the effects on cultured astrocytes of 1-h exposure to the 1-mT DC MF. The levels of hsp25, hsp60, and actin were different in serum-deprived cells and in serum-maintained cells. However, no significant differences in any protein levels were seen between the control and MF-exposed cells of each group (i.e., within the serum and serum-deprived groups). Because of the generally low level of protein expression shown by serum-deprived astroglial cells, it was decided to continue the study with such cells. The absence of any

Table 1  
Primary antibodies

Antibody	Anti-hsp25	Anti-hsp60	Anti-hsp70	Anti-actin	Anti-GFAP
Dilution	1:4000	1:1500	1:3000	1:1000	1:1000
Type	PAB	MAB	MAB	MAB	MAB
Supplier	Stressgen	Stressgen	Sigma	Chemicon	Sigma
Clone	Polyclonal	LK2	BRM-22	C4	G-A-5

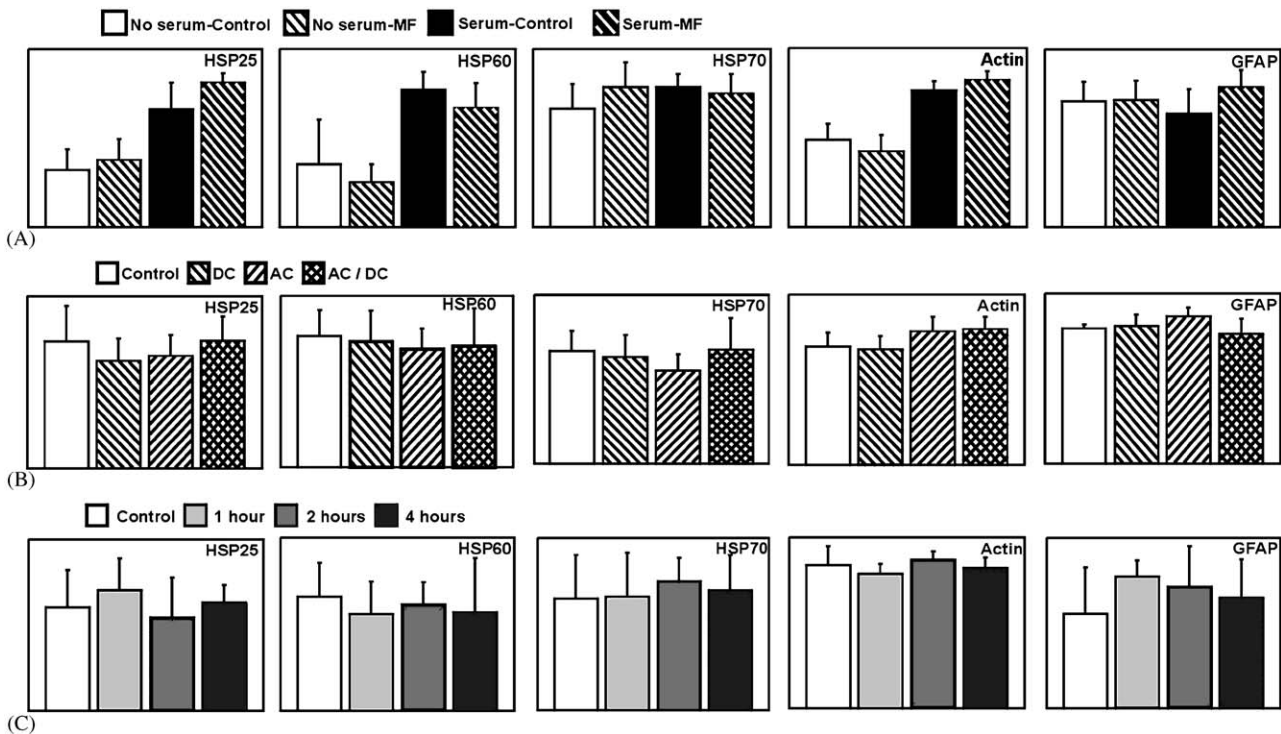


Fig. 3. Protein levels. Bars represent means ± SEM in arbitrary units; *n* = 3 (in triplicate). (A) Effect of 1-mT static MF exposure for 1 h under serum and serum-deprived conditions. (B) Effect of 1-mT static (DC), sinusoidal (AC), and combination (AC/DC) MF exposure for 1 h. (C) Effect of 1-mT AC/DC MF exposure for 1, 2, and 4 h.

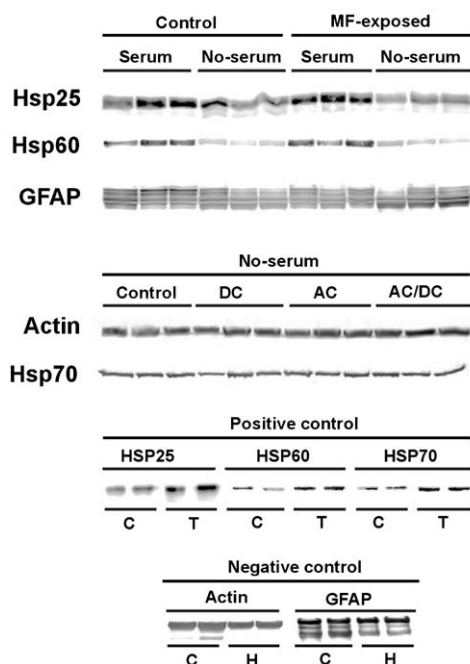


Fig. 4. Examples of Western blots for hsp25, hsp60, hsp70, actin, and GFAP under different experimental conditions. Upper area: hsp25, hsp60, and GFAP Western blots showing the effect of 1-mT DC MF exposure for 1 h on serum and serum-deprived astroglial cells. Middle area: hsp70 and actin Western blots for the experiment on the effects of 1-mT static (DC), sinusoidal (AC), or combined (AC/DC) MF exposure for 1 h. Lower area: a positive control including Western blots of control (C), heat-shocked (T) (42 °C for 45 min), and hyperammonemic (H) (5 mM NH<sub>4</sub>Cl for 4 days) astroglial cells.

effect under the DC MF regimen was the reason for testing the 50-Hz AC MF and AC/DC MF.

Fig. 3B shows the effects on the astroglial cells of 1 h of exposure to 1-mT 50-Hz AC, 1-mT DC, and 1-mT AC/DC MFs. No significant differences were seen between the control and the exposed groups with respect to any of the protein levels tested. However, for hsp25, hsp60, hsp70, and GFAP, cells exposed to AC/DC MFs showed a greater standard deviation than those exposed to AC or DC MFs. The effect of longer exposures to AC/DC MFs was therefore investigated.

Fig. 3C shows that exposure of the cells to a 1-mT AC/DC MF for 1, 2, and 4 h had no significant effect on the levels of the proteins studied. Similarly, the continuous exposure of astroglial cells for 11 days (starting 24 h after the cell plating) to a 1-mT AC/DC MF had no significant effect on cell proliferation (Fig. 2C). Neither were any structural differences (size and morphology) observed between the exposed and control cells in the different experimental regimens. Fig. 4 shows some immunoblot images of the analyzed proteins.

A positive control (Fig. 4) was performed for HSPs, with cells subjected to 42 °C for 45 min; a negative control was also developed for GFAP and actin, with

cells subjected to 5 mM ammonium chloride for 4 days. A significant (data not included) increase of hsp25, hsp60, and hsp70 levels was detected in these heat-shocked astrocytes. A significantly lower level of GFAP and actin was also observed in astrocytes after hyperammonemia.

#### 4. Discussion

The nervous system is one of the systems most sensitive to MFs, and glia seem to be the main cells responsible for neural reactions to them (see review of Zhadin, 2001). Occupational exposure to EMFs has been reported to be associated with a higher risk of glioma (Kheifets et al., 1995) and astrocytoma (Theriault et al., 1994). The possibility that MF exposure might enhance the effect of certain chemicals potentially responsible for gliomas has also been suggested (Navas et al., 2002). However, the present study shows that the acute exposure of cultures of astroglial cells to 1-mT AC, DC, and AC/DC MFs leads to no significant effects on the cytoskeletal (actin and GFAP) and stress proteins (hsp25, hsp60, and hsp70) and that chronic MF exposure does not seem to induce any changes in their proliferation.

The cytoskeleton is the main system responsible for cell shape. Strong MFs affect the cytoskeleton of smooth muscle cells (Iwasaka et al., 2003) and induce changes in glioblastoma cell orientation, probably due to the arrangement of microtubules (Hirose et al., 2003). In fact, when microtubules are assembled in an MF, they align their long axis parallel to it (Bras et al., 1998). Actin microfilaments have also been proposed to represent a cellular interaction site for MFs (Gartzke and Lange, 2002). Reorganization of cytoskeletal components—especially actin microfilaments—has been described in human B lymphoid cells after exposure to low intensity MFs (Santoro et al., 1997; Lisi et al., 2000), and a differential actin distribution has been shown in human keratinocytes after exposure to a 2-mT sinusoidal MF (Manni et al., 2002). However, in the present study no significant differences were found in the actin and GFAP (intermediate filament) content in exposed cells, nor were any changes in cell size or shape observed. The literature reports similar results for human cell cultures exposed to a 0.5-mT AC MF (Supino et al., 2001; Santini et al., 2003), with no changes in the intracellular distribution or amount of cytokeatin (intermediate filament) (Supino et al., 2001). It is difficult to explain these controversial results, but the intensity and type of MF and the cell type must be taken into account. It should also be remembered that the morphological effects of MFs on adherent cells could be very different from those on nonattached cells

and that cytoskeletal reorganizational changes may not be detected at the level of cytoskeletal proteins.

The first studies on the effects of MF exposure on stress proteins were those of Goodman and co-workers (see review of Goodman and Blank, 1998), who showed that cells do respond to MFs as an environmental stress. Increased stress protein levels were shown by Pipkin et al. (1999) in response to MF exposure in HL-60 cells in culture, and hsp16 levels were found to rise in *Caenorhabditis elegans* (Miyakawa et al., 2001). The contribution of HSF1 (heat shock factor 1) (Lin et al., 1997) and *myc* expression (Lin et al., 1998) to the induction of hsp70 expression in response to MF exposure has also been shown. Indeed, a clear upregulation of hsp70 promoters (Junkersdorf et al., 2000), the existence of a magnetic field-responsive domain in the human hsp70 promoter (Lin et al., 2001), and an induction of the hsp70 genes greater than that of hsp27, hsp60, and hsp90 genes (Tokalov and Gutzeit, 2004) have been seen after MF exposure. Nevertheless, contradictory results regarding the effect of MF exposure on stress proteins have also been documented. MF exposure did not alter the expression of hsp23 and hsp70 in embryonic cell cultures of *Drosophila melanogaster* (Koundakjian et al., 1996), or hsp70 production in HL60 cells (Miyakoshi et al., 2000; Morehouse and Owen, 2000), or hsp70 and hsp90 in 34i cells (Kang et al., 1998), or hsp25, hsp60, and hsp70 levels in cultured astroglial cells in the present study. Field intensity, frequency, and exposure time should be considered as explanations of these controversial results. The MF intensities used in the studies cited above ranged from weak ( $<100\ \mu\text{T}$ ) to medium (400 mT to  $100\ \mu\text{T}$ ) values; but inductive and noninductive effects of MF exposure on stress protein expression were described for the whole range. However, it seems that short-term exposures are more effective at inducing stress protein expression than are long-term exposures (Miyakoshi et al., 2000; Di Carlo et al., 2001).

Because of its relation with the appearance of cancer, the effect of MFs on cell proliferation has been widely analyzed. Some studies suggest that MF exposure could affect cell proliferation “in vitro” (Schimmelpfeng and Dertinger, 1997) as well as “in vivo” (Fedrowitz et al., 2002). However, MF exposures comparable to those used in the present study led to no differences in cell proliferation (Supino et al., 2001; Yoshizawa et al., 2002). Similar results have been obtained using stronger MFs (Wiskirchen et al., 1999, 2000). Further, MFs appear to have no effect on the basal rate of cell mortality (Boland et al., 2002) or on oncogene (*c-myc*, *c-fos*, *c-jun*) expression (Miyakoshi et al., 1996; Harrison et al., 1997; Jahreis et al., 1998; Yomori et al., 2002). In a study with arrays containing cDNAs for 588 cancer genes, Loberg et al. (2000) found no relationship between MF exposure and differential gene expression.

Although MF exposure induces very different biological effects, most studies report inconsistent and sometimes controversial results. However, the type of field, the duration of exposure, and the intensities and frequencies used in these protocols were very different, and this might explain most discrepancies (Berg, 1999). In addition to the well-known intensity and frequency window effect (the biological effects of MFs appear within specific narrow ranges of field intensity and frequency), the species-dependent effect (Levin and Ernst, 1997) and the cell-dependent effect (Buemi et al., 2001) should also be taken into account. Different sensitivities to MF exposure have been demonstrated recently in different strains of Sprague–Dawley rats (Fedrowitz et al., 2004), suggesting that the genetic background may play a pivotal role in the effects of MF exposure. However, the involvement of MF-induced apoptosis in the MF intensity window was discarded in a recent study by Tokalov and Gutzeit (2004). In the literature, the MF intensities used have been very different, ranging from  $\mu\text{T}$  to T, although usually from  $\mu\text{T}$  to mT. In order to reproduce the magnetic flux conditions similar to those found in some works (electricity generation, resistance welders, crack detection systems, tape eraser) (Cooper, 2002) or similar to those produced by home appliances (Barnes et al., 1987, cited in US Congress, Office of Technology Assessment, 1989), we exposed rat astroglial cultured cells to acute (1, 2, and 4 h) and chronic (11 days) 1-mT AC, DC, and AC/DC MFs and found no statistically significant effect of MF exposure on cytoskeletal or stress protein expression and on astroglial proliferation in culture. In conclusion, these results suggest that these types of MF have little or not effect on the expression of cytoskeletal and stress proteins and on proliferation in cultured astroglial cells.

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