

Effects of 60-Hz Magnetic Fields on DNA Damage Responses in HT22 Mouse Hippocampal Cell Lines

Gil-Im Mun¹ · Seungwoo Lee² · Nam Kim² · Yun-Sil Lee^{1*}

Abstract

Previously, we investigated extremely low-frequency magnetic fields (ELF-MFs) on diverse DNA damage responses, such as phosphorylated H2AX (γ H2AX), comet tail moments, and aneuploidy production in several non-tumorigenic epithelial or fibroblast cell lines. However, the effect of ELF-MF on DNA damage responses in neuronal cells may not be well evaluated. Here, we investigated the effects of ELF-MF on the DNA damage responses in HT22 non-tumorigenic mouse neuronal cells. Exposure to a 60-Hz, 2 mT ELF-MF did not produce any increased γ H2AX expression, comet tail moments, or aneuploidy formation. However, 2 mT ELF-MF transiently increased the cell number. From the results, ELF-MF could affect the DNA damage responses differently, depending on the cell lines.

Key Words: Aneuploidy, Cell Lines, Comet Tail, Extremely Low Frequency, γ H2AX.

I. INTRODUCTION

Extremely low-frequency magnetic fields (ELF-MF) under 50/60 Hz, which are generated by sources, such as electrical appliances or power lines, have created social concerns about possible adverse effects on human health. However, the carcinogenic potential of ELF-MF has been assessed experimentally in various model systems with inconsistent outcomes. Reviews of *in vivo* and *in vitro* laboratory studies on ELF-MF [1–6] have indicated contradictory results from genotoxic endpoints, such as chromosome aberrations, micronuclei, sister chromatid exchange, and DNA strand breakage. These results included increased DNA damage by ELF-MF under specific *in vitro* and *in vivo* conditions [7, 8], yet no such effects were reported in the other studies [9]. These inconsistent experimental data may be due to the differences in the equipment used to generate magnetic fields, exposure protocols, and bio-

logical materials, such as cell lines, animal species, strain, and age. Especially, in the case of *in vitro* studies, cell-line differences, such as from organs of different origin, may be a key point for inconsistent results. We demonstrated previously that a 1-mT ELF-MF alone did not induce cellular transformation in NIH3T3 mouse fibroblast cells [10], nor were there comet tail moments [11], micronucleus production [12], aneuploidy formation [13], or γ H2AX formation [14] in the mouse embryonic fibroblast NIH3T3 cells, WI38 human lung fibroblasts, or L132 human lung epithelial cells. However, a 2-mT ELF-MF increased γ H2AX formation [14], even though aneuploidy formation was not altered [13]. In an extension of this study, we here have used another cell line from organs of different origin, and which consists of non-tumorigenic mouse neuronal cells, HT22. These were examined for DNA damage responses such as comet tail moments, γ H2AX formation and aneuploidy formation after exposure to a 2-mT ELF-MF.

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II. MATERIALS AND METHODS

1. ELF Magnetic Field Exposure System

The equipment for ELF-MF generation was designed and constructed by the Korea Electrotechnology Research Institute (KERI, Changwon, Korea) and has been described previously in detail [10] (Fig. 1).

2. Cell Culture

HT22 mouse hippocampus neuronal cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-Invitrogen, Paisley, Scotland), and supplemented with 10% fetal bovine serum (HyClone, Thermo Fisher Scientific, Logan, UT, USA) at 37°C in an incubator with a humidified atmosphere of 95% air and 5% CO₂.

3. Exposure to ELF-MF

Cells (7.0×10^5) in 100 mm cell culture dishes were placed in the exposure chamber and subsequently exposed to a 60-Hz ELF-MF at 0 mT or 2 mT. Over the exposure time, the temperature in the chamber was maintained at 37°C±0.3°C by circulating water, and the temperature of the culture medium was monitored at 2-hour intervals. Positive controls were exposed to gamma radiation doses (10 Gy as a single dose), which were generated by a ¹³⁷Cs gamma-ray source (MDS Nordion, Ottawa, ON, Canada) at a dose rate of 5 Gy/min. Control cells were treated in the other incubator under the same experimental procedures, with the exception of ELF-MF exposure.

4. Immunoblotting

After ELF-MF exposure, equal amounts of protein (100 µg)

were dissolved in a lysis buffer. The samples were boiled for 5 minutes, and proteins were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to nitrocellulose membranes. After blocking with 5% skim milk in phosphate-buffered saline with Tween-20 (PBS-T), the membranes were incubated with γH2AX antibody (1:1000 dilution; EMD Millipore, Billerica, MA, USA) and β-Actin antibody (1:1000 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) for 18 hours at 4°C, washed with 1X PBS-T, and incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology). Immunoreactive bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific). The relative protein-band intensity was calculated from densitometric scans (MultiGauge version 3.0; Fujifilm, Tokyo, Japan) of immunoblots, with the control values set to 1 after normalization with β-Actin as an internal density control. Data were collected from duplicate samples and more than three independent experiments.

5. Comet Assay

The Comet Assay (Trevigen Inc., Gaithersburg, MD, USA) was performed according to the manufacturer's protocol. HT22 cells were seeded at a density of 7×10^5 cells/dish in 100-mm cell culture dishes. The 2-mT ELF-MF exposure times were 4 hours and 16 hours. Cells were resuspended in ice-cold PBS at a concentration of 1×10^5 cells/mL. An aliquot of 25 µL of cells (1×10^5 cells/mL) was added to 250 µL of 1% low-melting agarose, which was kept at 42°C. One hundred microliter aliquots were immediately pipetted and evenly spread over an area of the comet slides. The slides were incubated in the dark for 10 minutes at 4°C to accelerate gelling of the agarose discs, and then were transferred to a pre-chilled lysis solution (Trevigen Inc.) where they were kept for 60 minutes at 4°C. A denaturation step was performed in an alkali solution (300 mM NaOH, 1 mM EDTA, pH > 13) at 4°C for 10 minutes, in the dark. The slides were then transferred to a pre-chilled alkaline electrophoresis solution with pH > 13 (300 mM NaOH, 1 mM EDTA) and subjected to electrophoresis at 1 V/cm and 300 mA for 30 minutes. At the end of the electrophoresis, the slides were washed with a neutralizing buffer (0.4 M Tris-HCl, pH = 7.4), immersed in ice-cold 70% ethanol at room temperature for 5 minutes, and air dried. The DNA was stained with ethidium bromide for 20 minutes in the refrigerator, after which the slides were analyzed immediately using the TriTek CometScore Freeware v1.5 image analysis software. Detection was made for Olive tail moments, where the tail moment is defined as the product of the tail length and the fraction of total DNA in the tail. A tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail

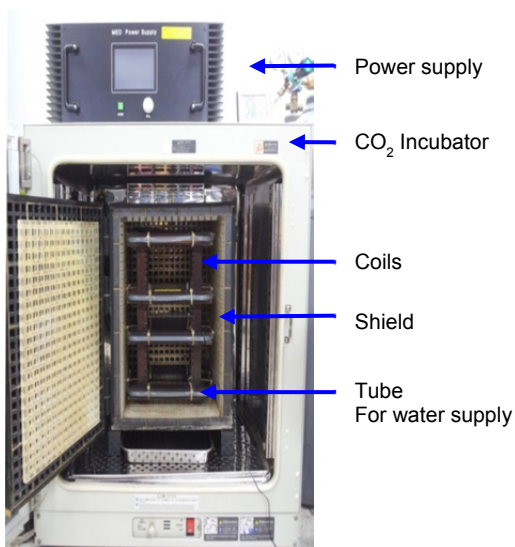


Fig. 1. A 60-Hz extremely low-frequency magnetic field exposure system for *in vitro* studies.

length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail). Each assay was performed in triplicate and in at least three independent experiments.

6. Flow Cytometry Analysis

Cell cycle distributions were analyzed using propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) staining, followed by flow cytometry. Cells were seeded at a density of 1×10^6 cells/dish in 100-mm cell culture dishes and incubated overnight. After exposure to a 2-mT ELF-MF for 6, 12, and 24 hours, cells were collected by trypsinization and harvested by centrifugation at 1,300 rpm for 3 minutes. Next, cells were fixed with 70% cold ethanol at -20°C overnight, washed with PBS, treated with RNase A (1 mg/mL; Sigma-Aldrich), and stained with propidium iodide (50 $\mu\text{g/mL}$). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA); and the data were analyzed with BD CellQuest Pro Software (BD Biosciences).

7. Cell Number Counting

HT22 cells were seeded at a density of 7×10^4 cells/dish in 60-mm cell culture dishes. After 12 hours, cells were exposed to a 2-mT ELF-MF. Exposure times were 6 hours and 12 hours. Cell numbers were counted after trypan blue staining. Control cells were treated in the other incubator under the same experimental procedures, with the exception of the ELF-MF exposure.

8. Statistical Analysis

Data are expressed as the mean \pm standard deviation (SD). Statistical significance was determined using Student's *t*-test. Statistically significant differences were assumed at $p < 0.05$.

III. RESULTS AND DISCUSSION

Numerous experimental studies have attempted to examine the carcinogenic potential of ELF-MFs. Even though a number of studies have reported DNA damage effects from ELF-MFs, the overall results remain inconclusive [9, 10, 15–19]. The main reason for this inconsistency was the use of different cell lines.

Previously, we investigated the effects of ELF-MFs on DNA damage responses, such as cellular transformation [10], comet tail moments [11], micronuclei formation [12], aneuploidy formation [13], and γH2AX expression [14]. These investigations were performed using non-tumorigenic epithelial or fibroblast cell lines. Among them, γH2AX expression was significantly altered by a 2-mT ELF-MF. In this study, we examined these DNA damage effects using a different cell line, HT22 mouse neuronal cells.

Expression of γH2AX in the HT22 cells was compared after

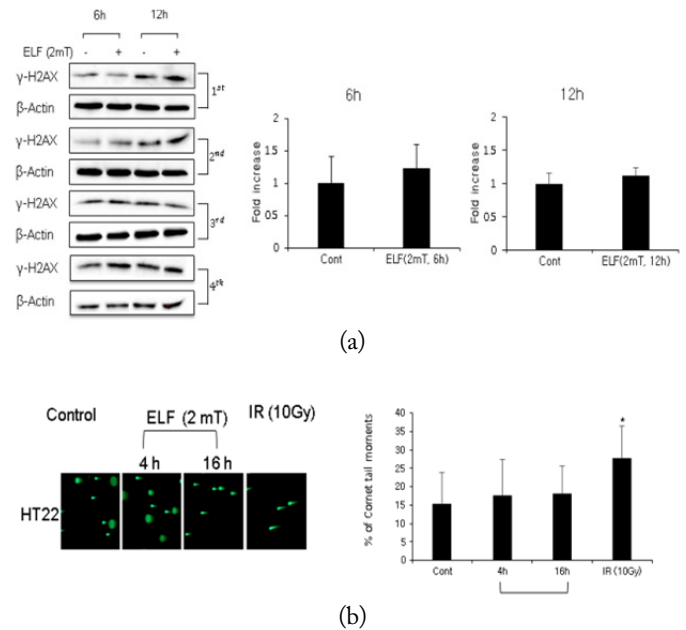


Fig. 2. Effects of extremely low-frequency magnetic field (ELF-MF) exposure on γH2AX expression and comet tail formation. (a) HT22 cells were exposed to a 2 mT ELF-MF. After 6 hours and 12 hours, cells were harvested and Western blotting was performed, using a specific antibody against γH2AX (left). The relative band intensity of γH2AX expression was calculated from densitometric scans of the blots with the control values set at 1. Protein expression was normalized to $\beta\text{-actin}$. Data are expressed as mean \pm SD from four independent experiments (right). (b) Cells (7.0×10^5) were placed in 100 mm cell culture dishes. HT22 cells were exposed to a 2-mT of ELF-MF. After 4 hours and 16 hours, cells were harvested. For a positive control, the cells were exposed to 10 Gy ionizing radiation (IR). Data are expressed as mean \pm SD from four independent experiments.

6 hours and 12 hours of exposure to a 2-mT ELF-MF. However, ELF-MF exposure did not result in an increased expression (Fig. 2(a)), nor was an increase in olive tail moments in the comet assay detected (Fig. 2(b)). Moreover, at 6, 12, and 24 hours of ELF-MF exposure at strengths of 2 mT, ELF-MFs did not alter the distributions of G2/M-arrested cells (before the cell cycle stage of aneuploidy), or aneuploid cells. Similarly, ELF-MF did not induce any increased apoptosis, which is represented by the subG1 population (Table 1), even though some proliferation ability was shown at 6 hours of ELF-MF exposure (Fig. 3).

Our previous study showed that exposure to a 1-mT ELF-MF for a maximum of 24 hours did not produce any increase in MN formation or comet tail production in several cell lines, including lung epithelial and fibroblast cells. Moreover, when we examined γH2AX , a 2-mT ELF-MF significantly increased γH2AX , as assayed by both Western blotting and visualization of foci formation [14]. However, in this study, when we used a

Table 1. Distribution of various cell cycle stages in HT22 cells after 2 mT ELF-MF exposure

	>4N (%)	<i>p</i> -value	G2/M (%)	<i>p</i> -value	SubG1 (%)	<i>p</i> -value
Cont	3.65±1.46		21.57±1.69		6.54±3.56	
6 hr	2.72±0.43	0.24	22.22±0.94	0.49	3.69±0.87	0.15
12 hr	3.34±0.52	0.70	23.87±2.20	0.05	3.79±0.53	0.16
24 hr	3.08±0.91	0.49	21.08±0.96	0.60	4.05±0.88	0.20

HT22 cells were exposed to a 2-mT ELF-MF. After 6, 12, and 24 hours, cells were harvested and analyzed using a flow cytometer. Data are expressed as mean ± SD from five independent experiments. ELF-MF = extremely low-frequency magnetic field.

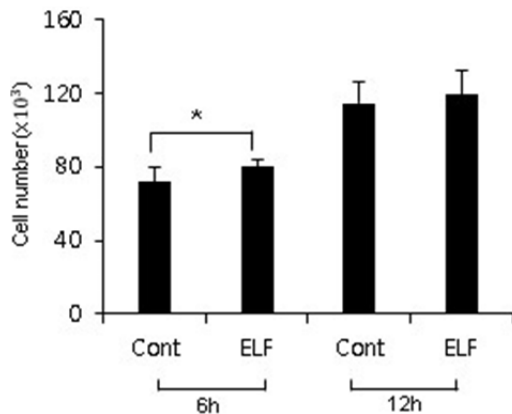


Fig. 3. Cell number of HT22 cells after extremely low-frequency magnetic field (ELF-MF) exposure. HT22 cells were exposed to a 2-mT ELF-MF. After 6 hours and 12 hours, cells were counted using trypan blue. Data are expressed as mean ± SD from three independent experiments.

different cell system, the neuronal cell line, the effects were different; γ H2AX expression was not altered by 2 mT ELF-MF in HT22 neuronal cells.

Different sensitivity for induction of γ H2AX might be mediated by different origins of cell lines; that is, epithelial/fibroblasts vs. neuronal cells. Another possibility for different sensitivity for γ H2AX induction is species difference; epithelial and fibroblast cells that were used in previous studies were from a human, while neuronal cells in this study were from a mouse.

The γ H2AX assay is capable of detecting DNA damage at a 100-fold higher sensitivity than that of the comet assay [20]. Our previous study showed that exposure to a 1-mT ELF-MF for a maximum of 24 hours did not show any increase in MN formation [12] or comet tail production in several cell lines [11]. However, when we examined γ H2AX, a 2-mT ELF-MF sig-

nificantly increased γ H2AX [14]. It should be noted that a 2-mT ELF-MF induced γ H2AX in certain cell lines.

The magnetic flux intensity (2 mT) was selected on the basis of Korean exposure guidelines, and this magnetic flux intensity was 2–10 times higher than the reference levels proposed by the International Commission on Non-Ionizing Radiation Protection (2010) for occupational exposure (1 mT) and for exposure of the general public (200 μ T), respectively.

From the results, we suggest that future experiments should be carried out in a systematic manner to guarantee the reliability of the results.

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