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Original Article

The Frequencies of Micronuclei Induced by Cisplatin in Newborn Rat Astrocytes Are Increased by 50-Hz, 7.5- and 10-mT Electromagnetic Fields

Yuichi MIYAKOSHI[1], Hayato YOSHIOKA[1], Yoshimitsu TOYAMA[1], Yuji SUZUKI[1] and Hidesuke SHIMIZU[1]

[1]*Department of Public Health and Environmental Medicine, The Jikei University School of Medicine, Tokyo, Japan*

Abstract

Objectives: Epidemiological studies have suggested that exposure to environmental and occupational electromagnetic fields (EMFs) contribute to the induction of brain tumors, leukemia, and other neoplasms. The aim of this study was to investigate the genotoxic effects of exposure to 50-Hz EMFs, and of co-exposure to cisplatin, a mutagen and carcinogen, and 50-Hz EMFs, using an in vivo newborn rat astrocyte micronucleus assay.

Methods: Three day-old male Sprague-Dawley rats were co-exposed to 50-Hz EMFs and 1.25 or 2.5 mg/kg of cisplatin. Brain cells were dissociated into single cells and cultured for 96 hours, then stained with acridine orange and an antibody against glial fibrillary acidic protein. The frequency of micronucleated astrocytes was counted with a fluorescent microscope.

Results: The frequency of micronuclei was not increased in rat astrocytes exposed to EMFs alone. However, the frequencies of micronuclei in co-exposure to 2.5 mg/kg cisplatin and EMFs (7.5- and 10-mT) were significantly increased, compared with those in exposure to 2.5 mg/kg cisplatin alone (sham-exposure, 0-mT EMFs) for 72 hours ($p<0.01$).

Conclusion: Exposure to EMFs alone did not have a genotoxic effect but co-exposure to EMFs increased the genotoxic activity induced by cisplatin. Our findings suggest that EMFs enhance the genotoxic effects of cisplatin.

Key words: electromagnetic fields, genotoxicity, astrocyte, cisplatin, micronucleus test

Introduction

Recently the machines that generate electromagnetic fields (EMFs) have come to be widely used in research (e.g. nuclear magnetic resonance systems: NMR and electron spin resonance system: ESR), in medicine (e.g. magnetic resonance imaging system: MRI), in industry (e.g. in aluminum plants and in magnet production), and in electric appliances in homes and offices (e.g. personal computers and cellular phones). Subsequently we have an increased chance of exposure to EMFs. Epidemiological studies have suggested that exposure to environmental and occupational EMFs contribute to the induction of brain tumors, leukemia, and other neoplasmas (1, 2). The genotoxic effects of EMFs, using microbial system and cultured cells etc., are well known, but there have been no reports about the genotoxicity induced by the co-exposure to cisplatin and EMFs in astrocytes in vivo study (3, 4). The micronucleus assay has been used as a short-term screening system to detect mutagens and carcinogens (5). The aim of this study was to investigate the genotoxic effects of exposure to 50-Hz, 10-mT EMFs alone, and of co-exposure to cisplatin, a mutagen and carcinogen, and 50-Hz, 1-, 5-, 7.5- and 10-mT EMFs, using an in vivo newborn rat astrocyte micronucleus assay.

Materials and Methods

Chemicals

Penicillin-streptomycin and a trypsin solution were obtained from Invitrogen Corp. (Carlsbad, CA, USA). DNase I was obtained from Roche Diagnostics GmbH (Mannheim, Germany). A rabbit polyclonal antibody against cow glial fibrillary acidic protein (GFAP) was obtained from DakoCytomation California (Carpinteria, CA, USA). A rhodamine-conjugated swine polyclonal anti-rabbit immunogloblin was obtained from Dako-

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Reprint requests to: Yuichi MIYAKOSHI
Department of Public Health and Environmental Medicine, The Jikei University School of Medicine, 3-25-8 Nishishinbashi, Minato-ku, Tokyo 105-8461, Japan
TEL: +81(3)3433-1111 (ex. 2266), FAX: +81(3)5472-7526
E-mail: miyakoshi@jikei.ac.jp

Cytomation Denmark A/S (Glostrup, Denmark). Dulbecco's phosphate-buffered saline (PBS), fetal bovine serum (FBS), minimum essential medium with Earle's salts (MEM), poly-L-lysine, and Triton-X were obtained from Sigma-Aldrich Co., (St. Louis, MO, USA). Acridine orange was obtained from Dojindo Laboratories (Kumamoto, Japan). Cisplatin was obtained from Maruko Pharmaceutical Co., Ltd., (Nagoya, Japan).

Animals and EMF exposure

Three-day-old male Sprague-Dawley rats were used for the in vivo newborn rat astrocyte micronucleus assay (Charles River Japan, Yokohama, Japan). The rats were kept in a clean room (constant temperature of 22–24°C, 45% to 55% humidity, with lights on 24 hours/day. The rats were given chow (CRF-1, Charles River Japan) and tap water ad libitum.

All animal experiments were performed in accordance with Animal Experiments Guidelines of The Jikei University School of Medicine.

The EMFs was generated with biological experimental magnetic field coils (IDX Co., Tokyo, Japan) and a function generator FG-275 (Kenwood TMI Co., Kanagawa, Japan) (Fig. 1). The coils were energized to generate horizontal sinusoidal EMFs of 50-Hz and 0- to 10-mT.

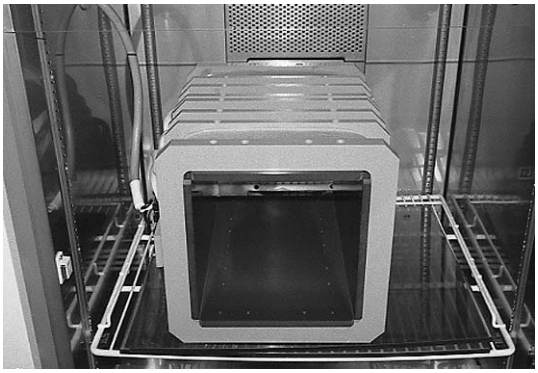

Fig. 1 The electromagnetic fields generator systems. The experimental magnetic fields coils to generate horizontal sinusoidal EMFs of 50-Hz, 10-mT (218×218×387 mm).

Ex vivo newborn rat astrocytes micronucleus assay

Rats were co-exposed to cisplatin and EMFs in the following four modes.

1) Rats were exposed to EMFs (50-Hz, 10-mT) alone for 24, 48, and 72 hours. The sham-exposure control rats were maintained in a coil without EMFs.

2) Cisplatin was administered to rats as a single intraperitoneal dose of 2.5 mg/kg, and physiological saline was used as a control solvent. Rats were co-exposed to EMFs (50-Hz, 10-mT) for 24, 48 and 72 hours and the sham-exposure control rats were maintained in a coil without EMFs.

3) Cisplatin was administered to rats as a single intraperitoneal dose of 1.25 or 2.5 mg/kg, and physiological saline was used as a control solvent. Rats were co-exposed to EMFs (50-Hz, 10-mT) for 72 hours, and the sham-exposure control rats were maintained in a coil without EMFs.

4) Cisplatin was administered to rats as a single intraperitoneal dose of 2.5 mg/kg, and physiological saline was used as a control solvent. Rats were co-exposed to 1-, 5-, 7.5- and 10-mT EMFs (50-Hz) for 72 hours, and the sham-exposure control rats were maintained in a coil without EMFs.

The whole brains were removed from Sprague-Dawley rats and incubated in PBS containing 0.25% trypsin and 40 mg/ml DNase for 30 minutes at 37°C, according to the method of Toga et al. (6). After inactivation of trypsin activity, the cell suspensions were centrifuged at 1000 rpm for 10 minutes. The cell pellet was resuspended in MEM containing 5% FBS, 5 mg/ml glucose, 100 U/ml penicillin, and 100 mg/ml streptomycin. The suspended brain cells were dissociated into single cells by gentle pipetting. The nucleated cells were cultured in MEM containing 5% FBS for 96 hours on poly-L-lysine coated 25-mm-diameter glass cover slips in a 5% CO_2 incubator at 37°C.

The cells growing on the coverslips were rinsed in PBS, and then were fixed with cold methanol for 10 minutes. The fixed cells were washed in PBS containing 0.1% Triton X-100, and then incubated with a rabbit polyclonal anti-cow GFAP antibody for 60 minutes at 37°C. The cells were washed in PBS, then were incubated with rhodamine-conjugated swine polyclonal anti-rabbit immunogloblin for 30 minutes at room

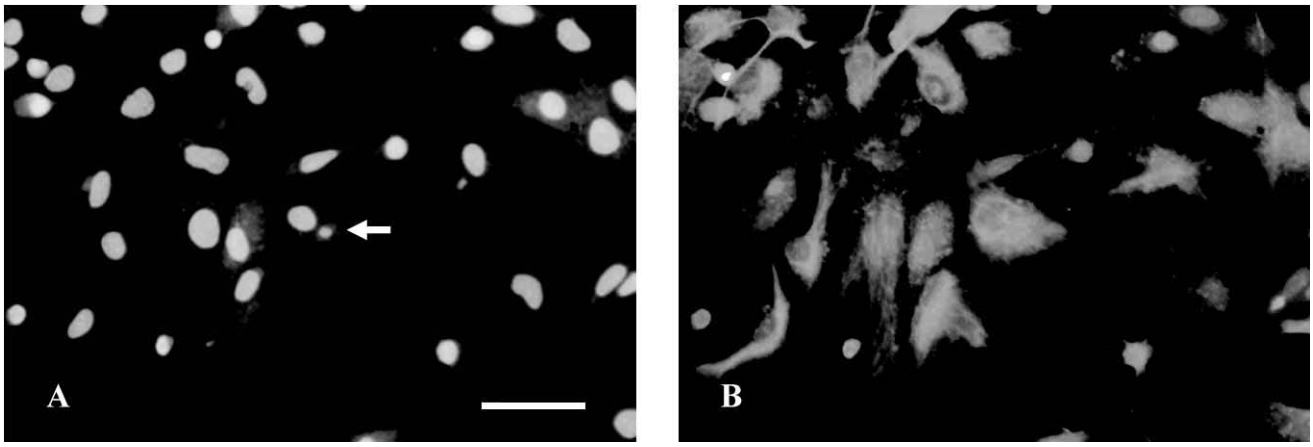

Fig. 2 Micronuclei in rat astrocytes. Nucleated cells were doubled labeled with acridine orange to identify nuclei and micronuclei (A), and with GFAP antibody to identify astrocytes (B). Arrowhead indicates micronuclei. Scale bar=50 μm.

temperature. To recognize main nuclei and micronuclei, the cells were stained with 1.25 mM acridine orange in distilled water for 5 minutes at room temperature. After the cells were washed in PBS, the cover slips were mounted on glass slides in PBS. Astrocytes were observed with a microscope (Olympus,. Optical Co., Ltd., Tokyo, Japan) equipped with phase-contrast, fluorescein, and rhodamine optics at 300x magnification. Astrocytes were identified as GFAP-positive nucleated cells (Fig. 2). The frequency of micronucleated astrocytes was determined by counting 1000 GFAP-positive nucleated cells (6, 7).

Micronucleated cells were identified with the following criteria: 1) counted cells should have normal cell morphology with cytoplasmic borders, 2) the diameter of the micronuclei should be no larger than one-third of the main nucleus, and 3) binucleated or polynucleated cells should be not scored (8).

Three rats were assayed in each group. The experimental results were analyzed statistically using the Kastenbaum-Bowman table (conditioned binomial probability test) (9).

Results

The frequency of micronuclei in the EMF alone group did not significantly increase compared to those in the sham-exposure group for 24, 48 and 72 hours (Fig. 3). Fig. 4 shows the frequency of micronuclei in the 2.5 mg/kg cisplatin and EMF-co-exposure group for 24, 48 and 72 hours. The frequency of micronuclei in the 2.5 mg/kg cisplatin and EMF-co-exposure group was 2.8 times higher at 48 hours ($p<0.01$) and 1.9 times higher at 72 hours ($p<0.01$) than that in the 2.5 mg/kg cisplatin-exposure group (sham-exposure group). And similar to Fig. 3, the frequency of micronuclei in the EMF alone group (EMF+physiological saline) did not significantly increase compared to those in the sham-exposure group (physiological saline alone) for 24, 48 and 72 hours. The frequency or micronuclei in the 2.5 mg/kg cisplatin-exposure group (sham-exposure group) was 2.0 times higher at 72 hours ($p<0.01$) than that in the same group at 24 hours. The frequency of micronuclei in the 2.5 mg/kg cisplatin and EMF-co-exposure group was 2.5 times higher at 48 hours ($p<0.01$) and 3.1 times higher at 72 hours ($p<0.01$) than that in the same group at 24 hours. The frequency of micronuclei was highest after 72 hours of EMF exposure in the 2.5 mg/kg cisplatin and EMF co-exposure group. Accordingly, the dose-response study of cisplatin with co-exposure to EMFs was done at 72 hours exposure. The frequency of micronuclei in the EMF-exposure groups was 2.0 times higher with a cisplatin dose of 1.25 mg/kg ($p<0.01$), and 1.9 times higher

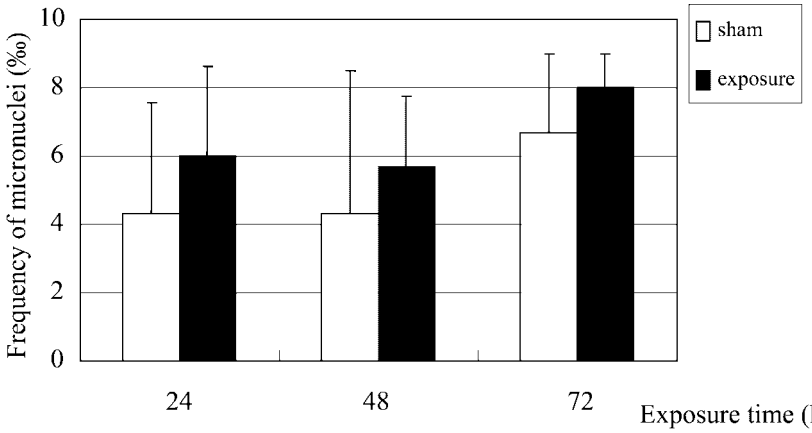

Fig. 3 Frequency of micronuclei in rat astrocytes with exposure to 10-mT EMFs for 24, 48 and 72 hours. In each group, n=3.

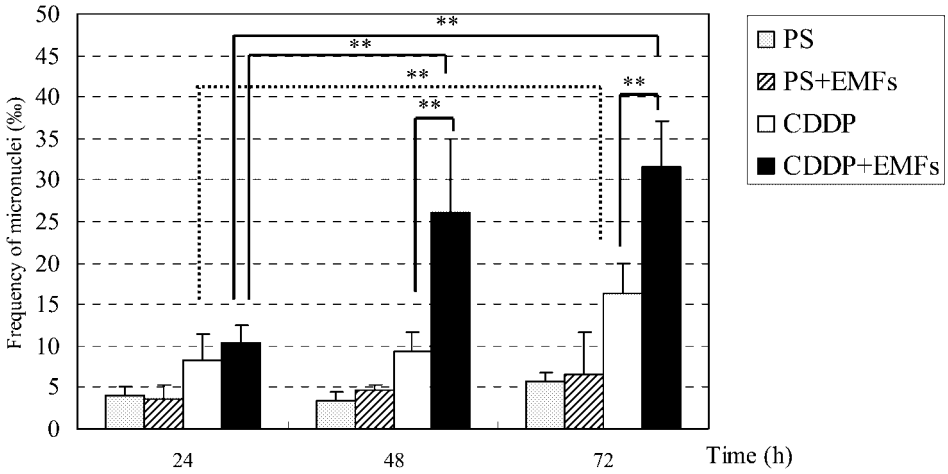

Fig. 4 Frequency of micronuclei in rat astrocytes induced by 2.5 mg/kg CDDP co-exposure to 10-mT EMFs for 24, 48 and 72 hours. PS: physiological saline, CDDP: cisplatin. In each group, n=3. **: $p<0.01$.

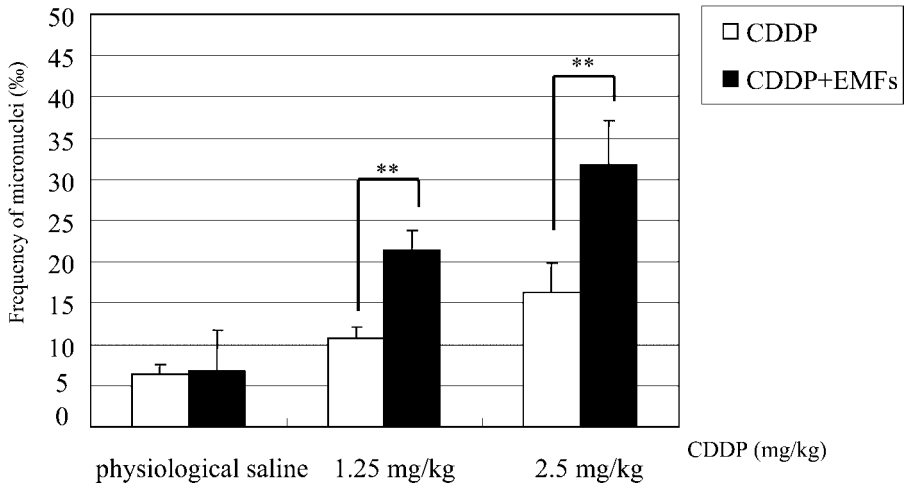

Fig. 5 Frequency of micronuclei in rat astrocytes after 72 hours' co-exposure to cisplatin and 10-mT EMFs in a dose-response study of cisplatin. In each group, n=3. **: $p<0.01$.

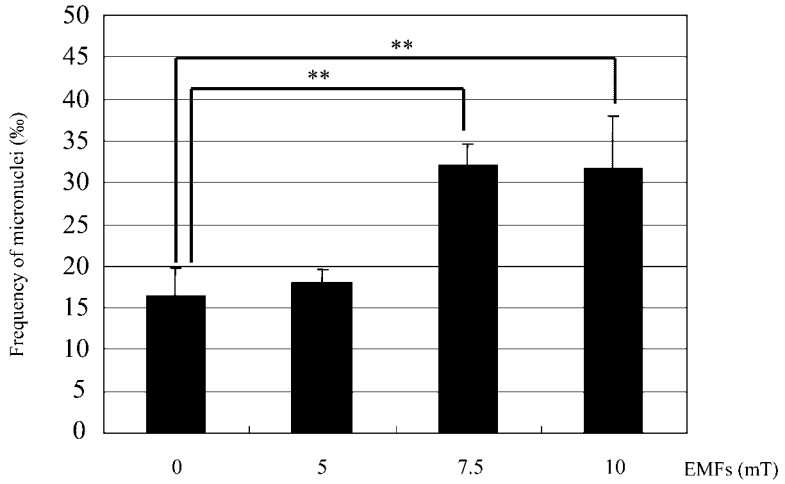

Fig. 6 Frequency of micronuclei in rat astrocytes after 72 hours' co-exposure to 2.5 mg/kg cisplatin and EMFs in dose-response study of EMFs. In each group, n=3. **: $p<0.01$.

with a cisplatin dose of 2.5 mg/kg ($p<0.01$), than the sham-exposure group (Fig. 5). The frequency of micronuclei was highest with a dose of 2.5 mg/kg, and the dose-response relationship of micronucleus frequencies was observed up to a dose of 2.5 mg/kg cisplatin in both the EMF-exposure and the sham-exposure groups. As with the dose-response study of cisplatin, the dose-response study of EMFs with co-exposure to cisplatin was performed for 72 hours' exposure. The frequency of micronuclei in the EMF-exposure group was 2.0 times higher at 7.5-mT ($p<0.01$) and 1.9 times at 10-mT ($p<0.01$) than in the sham-exposure group (Fig. 6). The frequency of micronuclei was highest at 7.5-mT, and the frequencies of micronuclei increased until an EMF strength of 10-mT.

Discussion

Our study has shown that micronuclei in astrocytes are not increased by exposure to 50-Hz, 10-mT EMFs alone, and that micronuclei induced by cisplatin are increased by co-exposure to 50-Hz, 7.5- and 10-mT EMFs.

Some studies have reported that co-exposure to EMFs promote the growth of tumors induced by 7,12-dimethylbenz (a) anthracene (DMBA) (10, 11). Loscher et al. have reported that female rats co-exposed to EMFs (50-Hz, 100-μT, 91 days) and orally administered 20 mg/body DMBA exhibited significantly more mammary tumors than did EMF-sham-exposed rats (10). Thun-Battersby et al. have reported that the development and growth of mammary tumors is facilitated in female rats exposed to EMFs (50-Hz, 100-μT, 13 weeks) and orally administered 10 mg/body DMBA (11). Mevissen et al. (12) have reported that exposure to EMFs (50-Hz, 50-μT, 6 weeks) increases the activity of ornithine decarboxylase (ODC), a key enzyme in the biosynthesis of polyamines that promotes cell proliferation, in rat mammary tissues. Exposure to EMFs significantly increases the activity of ODC in mammary tissue and the spleen. The increase in ODC produced by EMF exposure in mammary tissue is of a similar magnitude to that produced by treatment with 5 mg/body DMBA alone or by combined treatment with EMFs and 5 mg/body DMBA. Wei et al. have reported that EMFs (60-Hz, 30- to 120-μT, 3 to 72 hours) enhance the growth of human astrocytoma cells in vitro (13). Exposure to EMFs strongly potentiates the effect of the

muscarinic agonists carbachol and phorbol 12-myristate 13-acetate (13).

Micronuclei are induced in the cytoplasm through the formation of chromosomal fragments or remnants of chromosomes from cell division disturbed by clastogens or spindle poisons and are used to screen for genotoxicity (5). We have performed a micronucleus assay using primary cultured newborn rat astrocytes (7, 14, 15). However, the genotoxicity of EMFs has not been studied with an in vivo newborn rat astrocyte micronucleus assay.

Cisplatin is a widely used antitumor agent (16, 17). It reacts with DNA to form DNA adducts, which are DNA-intrastrand and DNA-interstrand cross-links. Cisplatin is both a mutagen and carcinogen (18–22).

Exposure to EMFs might promote the growth of chemically induced tumors, and it is suggested that they would have tumor-promoting or co-promoting effects (10). Several studies have shown that 50- or 60-Hz EMFs can promote or co-promote cancer development in cells or tissues exposed to carcinogens. These studies examined depression of pineal melatonin production, or function, disruption of calcium homeostasis, activation of oncogenes, impairment of immune system antitumor control mechanisms, and modulation of ODC activity (23–29). Loscher et al. (26) have suggested that a reduction in circulating melatonin levels is related to tumor promotion. Many reports have demonstrated that melatonin inhibits the growth of a wide range of cancers (30). Melatonin's oncostatic effects involve complex interactions with hormones, growth factors, cytokines, and their receptors as well as various signal-transduction pathways, cytoskeletal elements, and oncogenes (30). A reduction in melatonin may 1) increase proliferation of stem cells at risk, 2) stimulate proliferation of cancer cells, and 3) impair immune function (31, 32).

A direct effect of EMF exposure is increased expression of several oncogenes, including *c-myc*, in different cell systems (33–35). The proteins produced by oncogenes, such as *myc*, might facilitate progression of cells through the cell cycle and DNA synthesis in the S phase (36).

Tofani et al. (37) have suggested that platinum ions derived from cisplatin stimulate superoxide radical production and that EMF exposure enhances active oxygen production. Active oxygen species are produced by cisplatin at the cell membrane, which undergoes permeability changes that influence cellular drug intake. Tofani et al. have also found that the rate of conversion of cisplatin to reactive species that can bind to DNA is increased by localized production of free radicals.

Our study shows that the genotoxic activity is not increased by the exposure to EMFs alone and that the genotoxic activity of cisplatin is increased by the co-exposure to 50-Hz, 7.5- and 10-mT EMFs, and suggested that the threshold of genotoxicity induced by co-exposure to EMFs and cisplatin for 72 hours could be between 5-mT and 7.5-mT. We also previously reported the suppression or enhancement of the genotoxic effects in microbial systems, cultured cells and animals, the suppression of food and water consumption and body weight in mice, and the enhancement of metallothionein content and lipid peroxidation in mice, with exposure to strong static EMFs (0.15- to 11.75-T) alone or co-exposure to static EMFs and chemicals (38–43). Our previous studies suggested that the effects induced by strong static EMFs may be enhanced or suppressed by the stress reaction or cell cycle change. Our findings suggest three possible mechanisms for the increased genotoxicity induced by co-exposure to cisplatin and EMF: stress reactions, tumor-promoting effects and active oxygen species. On the other hand, the International Agency for Research on Cancer (IARC) (44) considers that extremely low-frequency magnetic fields are possibly carcinogenic to humans (Group 2B). In office and homes, the magnetic flux density may approach 2-mT from devices such as hair dryers, can openers and induction heating (IH) cooker (45). In industrial tools such as arc welders and induction furnaces, the magnetic flux intensity may be from 0.1- to 60-mT (45). We therefore have the possibility of exposure to several sources of EMFs at the mT densities of our study. Our study suggested that it is important to evaluate the risk of EMFs for humans.

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