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Fifty-hertz Electromagnetic Fields Increase the Frequencies of Micronuclei Induced by 5-Fluorouracil in Newborn Rat Astrocytes

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ABSTRACT

Objectives: Epidemiologic studies suggest that exposure to environmental and occupational electromagnetic fields (EMFs) contributes to the induction of brain tumors, leukemia, and other neoplasms. The aim of this study was to investigate the genotoxic effects of co-exposure to 5-fluorouracil (5-FU), a mutagen, 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 either 125 or 250 mg/kg of 5-FU. Brain cells were dissociated into single cells, cultured for 96 hours, then stained with acridine orange and an antibody against glial fibrillary acidic protein. The frequency of micronucleated astrocytes was determined with a fluorescent microscope.

Results: The frequency of micronuclei did not increase in rat astrocytes exposed to EMFs alone. However, the frequencies of micronuclei were significantly higher in rats exposed to both 250 mg/kg 5-FU and EMFs (10 mT) than in rats exposed to 250 mg/kg 5-FU alone (sham-exposure, 0-mT EMFs) for 72 hours ($p < 0.01$).

Conclusion: Exposure to EMFs alone does not have a genotoxic effect, but co-exposure to EMFs increases the genotoxic activity induced by 5-FU. Our findings suggest that EMFs enhance the genotoxic effects of 5-FU. (Jikeikai Med J 2005; 52: 115-22)

Key words: electromagnetic fields, genotoxicity, astrocyte, 5-fluorouracil, micronucleus test

INTRODUCTION

Devices that generate electromagnetic fields (EMFs) are widely used. Nuclear magnetic resonance and electron spin resonance systems in research and magnetic resonance imaging systems in medicine generate static magnetic fields. Personal computers and appliances in homes and offices generate extremely low frequency magnetic fields (3- to 3,000-Hz). Cellular phones generate ultrahigh frequency electromagnetic fields (300-MHz to 3-GHz). Consequently, exposure to EMFs has increased. We are also exposed to many chemicals from air pollution, food contamination, and other sources. In addition, co-exposure to EMFs and chemicals has increased. Epidemiologic studies suggest that exposure to environmental and occupational EMFs contributes to the development of brain tumors, leukemia, and other neoplasms[1,2].

Since a strong correlation between genotoxicity (mutations, chromosomal aberrations and DNA damages) and carcinogenicity was reported, determining the genotoxicity of chemicals has become more important for evaluating their carcinogenicity[3]. Therefore, many low-cost, short-term genotoxicity screening tests, such as the *Ames Salmonella* mutagenicity

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assay, chromosomal aberration tests, and the *in vivo* micronucleus assay, are widely used to detect the genotoxicity of chemicals and to identify carcinogens[4]. Micronuclei are chromosomal fragments or remnants formed in the cytoplasm during cell division by clastogens or spindle poisons and are used as indicators in genotoxicity screening systems. The micronucleus assay has been used as a short-term screening system to detect mutagens and carcinogens[5]. Genotoxicity tests have used to evaluate the carcinogenicity of EMFs, and the genotoxic effects of EMFs have been shown through studies with microbial systems and cultured cells. We have previously reported that exposure to 50-Hz EMFs increases the frequency of micronuclei induced by cisplatin, a mutagen and carcinogen, in newborn rat astrocytes[6].

5-Fluorouracil (5-FU) is a mutagen and carcinogen that is used as an anti-metabolite anticancer drug. In addition, 5-FU is widely used as a positive control chemical in genotoxicity assays. However, to our knowledge, the genotoxicity of co-exposure to 5-FU and EMFs in astrocytes has not been examined *in vivo*[7,8].

We are co-exposed to EMFs and chemicals in daily life. To evaluate the inducibility of brain tumors or carcinogenicity of astrocytes induced by EMFs, we investigated the genotoxic effects of co-exposure to 5-FU and 50-Hz, 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 bovine glial fibrillary acidic protein (GFAP) was obtained from Dako Cytomation California (Carpinteria, CA, USA). A rhodamine-conjugated swine polyclonal anti-rabbit immunoglobulin was obtained from Dako 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). 5-FU was obtained from Kyowa Hakko Kogyo Co., Ltd., (Tokyo, Japan).

Animals and EMF exposure

Three-day-old male Sprague-Dawley rats (Charles River Japan, Yokohama, Japan) were used for the *in vivo* newborn rat astrocyte micronucleus assay. The rats were kept in a clean room (at constant temperatures between 22°C and 24°C, and relative humidities between 45% and 55%), with lights on 24 hours a day. The rats were given food (CRF-1, Charles River Japan) and tap water ad libitum.

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

The EMFs were generated with biological experimental magnetic field coils (IDX Co., Tokyo, Japan) and a function generator (FG-275, Kenwood TMI Co., Kanagawa, Japan ; Fig. 1)[6]. The coils were energized to generate horizontal sinusoidal EMFs of 50 Hz and 0 to 10 mT.

Ex vivo newborn rat astrocytes micronucleus assay

The rats were co-exposed to 5-FU and EMFs in the following three modes.

1) 5-FU was administered to the rats as a single intraperitoneal dose of 250 mg/kg, and physiological saline was used as a control solvent. The rats were co-exposed to EMFs (50 Hz, 10 mT) for 24, 48, or 72 hours, and the sham-exposure control rats were maintained in a coil without EMFs.

2) 5-FU was administered to rats as a single intraperitoneal dose of either 125 or 250 mg/kg, and physiological saline was used as a control solvent. The 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.

3) 5-FU was administered to the rats as a single intraperitoneal dose of 250 mg/kg, and physiological saline was used as a control solvent. The rats were

co-exposed to 5-, 7.5- or 10-mT EMFs (50 Hz) for 72 hours, and the sham-exposure control rats were maintained in a coil without EMFs.

The entire brain was removed from each of the 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[9]. After trypsin had been inactivated, the cell suspensions were centrifuged at 1,000 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. Toga et al. have reported that 96 hours are required to observe and attach the astrocytes on cover slips and that rat astrocytes do not increase on cover slips in MEM containing 5% FBS for 96 hours[9]. 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.

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

The cells growing on the cover slips were rinsed in PBS, and then fixed with cold methanol for 10 minutes. The fixed cells were washed in PBS containing 0.1% Triton X-100, then incubated with a rabbit polyclonal anti-bovine GFAP antibody for 60 minutes at 37°C. The cells were washed in PBS and then incubated with rhodamine-conjugated swine polyclonal anti-rabbit immunogloblin for 30 minutes at room temperature. So that the main nuclei and the micronuclei could be easily recognized, 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)[6]. The frequency of micronucleated astrocytes was determined by counting 1000 GFAP-positive nucleated cells[9,10].

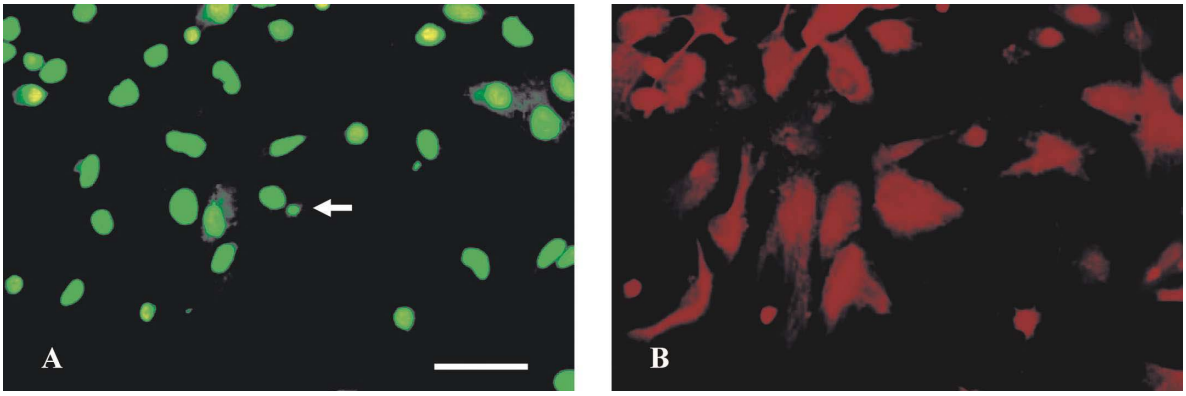

Fig. 2. Micronuclei in rat astrocytes. Nucleated cells were double-labeled with acridine orange to identify nuclei and micronuclei (A), and with an anti-GFAP antibody to identify astrocytes (B). Arrowhead indicates micronucleus. Scale bar=50 μm.

Micronucleated cells were identified with the following criteria: 1) normal cellular morphology with cytoplasmic borders, 2) micronuclei with a diameter no larger than one-third of the main nucleus, and 3) no binucleated or polynucleated cells[11].

Three rats were examined in each group. The experimental results were analyzed statistically using the Kastenbaum-Bowman table (conditioned binomial probability test)[12].

RESULTS

Frequency of micronuclei after co-exposure to 250 mg/kg 5-FU and EMFs in a time study

The frequency of micronuclei in the EMF alone group (physiological saline) did not differ significantly from that in the sham-exposure group (physiological saline) for 24, 48 and 72 hours (Table 1). The frequency of micronuclei at 72 hours was 1.6 times higher in the EMF-exposure groups ($p<0.01$) than in the sham-exposure group (Table 1). The frequency of micronuclei was highest at 72 hours, and the time-response relationship of micronucleus frequencies was observed for 72 hours in the EMF-exposure and the sham-exposure groups.

Frequency of micronuclei after co-exposure to 5-FU and EMFs in a dose-response study of 5-FU

In a time-study of EMFs with co-exposure to 5-FU, the frequency of micronuclei was highest after 72 hours of EMF exposure. Accordingly, the dose-response study for 5-FU was performed with co-exposure to EMFs for 72 hours. The frequency of micronuclei in the EMF-exposure groups was 1.9 times higher with a 5-FU dose of 125 mg/kg ($p<0.01$) and 1.6 times higher with a 5-FU dose of 250 mg/kg ($p<0.01$) than that in the sham-exposure group (Table 2). The frequency of micronuclei was highest with a dose of 250 mg/kg, and the dose-response relationship of micronucleus frequencies was observed until a 5-FU dose of 250 mg/kg in both the EMF-exposure and the sham-exposure groups.

Table 1. Frequency of micronuclei in rat astrocytes induced by co-exposure to 250 mg/kg 5-FU and 10-mT EMFs in a time study

Exposure time (hours)	Frequency of micronuclei			
	Sham exposure (‰±SD)		EMF exposure (‰±SD)	
	Physiological saline	250 mg/kg 5-FU	Physiological saline	250 mg/kg 5-FU
24	4.0±1.00	13.7±4.04	3.7±1.53	11.7±3.06
48	3.3±1.15	15.3±0.58	4.7±0.58	12.3±3.79
72	5.0±3.46	20.0±5.00	8.0±4.58	31.3±6.03*

In each group, $n=3$
*: $p<0.01$.
SD: standard deviation

Table 2. Frequency of micronuclei in rat astrocytes after 72 hours' co-exposure to 5-FU and 10-mT EMFs in a dose-response study of 5-FU

Dose of 5-FU (mg/kg)	Frequency of micronuclei	
	Sham exposure (‰±SD)	EMF exposure (‰±SD)
Physiological saline	5.0±3.46	8.0±4.58
125	9.3±1.53	18.0±8.19*
250	20.0±5.00	31.3±6.03*

In each group, $n=3$
*: $p<0.01$.
SD: standard deviation

Table 3. Frequency of micronuclei in rat astrocytes after 72 hours' co-exposure to 250 mg/kg 5-FU and EMFs in dose-response study of EMFs

EMFs (mT)	Frequency of micronuclei (‰±SD)
0 (Sham exposure)	20.0±5.00
5	19.3±2.08
7.5	25.7±2.89
10	31.3±6.03*

In each group, $n=3$
*: $p<0.01$.
SD: standard deviation

Frequency of micronuclei in dose-response study of EMFs with co-exposure to 5-FU

As with the dose-response study of 5-FU, the dose-response study of EMFs with co-exposure to 5-FU was performed for 72 hours of exposure. The frequency of micronuclei was 1.6 times higher in the EMF exposure groups at 10 mT ($p<0.01$) than in the sham-exposure group (Table 3). The frequency of micronuclei was highest at 10 mT, and the frequencies of micronuclei increased until an EMF strength of 10 mT.

Discussion

To evaluate the ability of EMFs to induce astrocytomas, we studied the genotoxicity induced by co-exposure to EMFs and 5-FU for the following reasons. 1) Numerous epidemiologic studies suggest that exposure to environmental and occupational EMFs contributes to the development of brain tumors, especially gliomas and astrocytomas, in studies[1,2]. 2) We are co-exposed to EMFs and chemicals from air pollutants, food contaminants, and other sources. 3) Genotoxicity is strongly correlated with carcinogenicity, and detecting the genotoxicity of chemicals has become more important for evaluating their carcinogenicity[3]. 4) The micronucleus assay is widely used as a short-term screening assay to detect the genotoxicity of chemicals[4]. 5) We performed an *in vivo* micronucleus assay in rat astrocytes, because astrocytes grow and increase during the neonatal period.

Genotoxicity can be investigated with micronuclei, which are induced in the cytoplasm through the formation of chromosomal fragments or remnants of chromosomes when cell division is disturbed by clastogens or spindle poisons[5]. We have performed a micronucleus assay using primary cultured newborn rat astrocytes[10,13,14]. However, to our knowledge, the genotoxicity of EMFs has not previously been studied with an *in vivo* newborn rat astrocyte micronucleus assay.

The antineoplastic effects of 5-FU require intracellular metabolic processes, mainly in the liver, and the presence of intracellular metabolites indicates antineoplastic effects[15]. 5-FU is an analogue of pyrimidine bases, and its metabolites include 5-fluorouridine 5′-monophosphate and 5-fluoro 2′-deoxyuridine 5′-monophosphate, which inhibit DNA biosynthesis and RNA functions[16]. 5-FU is a mutagen that produces chromosomal breakage in Chinese hamster ovary cells and increases micronuclei in the bone marrow of ICR mice[17]. 5-FU induces structural chromosomal aberrations in the first cell cycle from which micronuclei are directly derived in the second cell cycle[18].

Several studies in normal animals have shown that co-exposure to EMFs (50 Hz and 50-100 μT) and 7, 12-dimethylbenz (a) anthracene (DMBA) promotes the growth of chemically induced tumors and increases the activity in rat mammary tissues of ornithine decarboxylase (ODC), a key enzyme in the biosynthesis of polyamines which promotes cell proliferation[19-21].

A reduction in melatonin is related to tumor promotion[21-31]. Melatonin is believed to interact with the hormones, growth factors, cytokines, cytokine receptors, various signal-transduction pathways, cytoskeletal elements, and oncogenes. and the decrease of melatonin induced by EMFs would increase proliferation of stem cells and impair immune function[30,31].

Studies have been performed in carcinoma cell lines. A study in human astrocytoma cell line has shown that EMFs (60 Hz and 30-120 μT) promote cell growth and potentiates the effects of the muscarinic agonists carbachol and phorbol 12-myristate 13-acetate[32]. EMF exposure also increases expression and

proteins produced by the oncogenes *c-myc*[33-36].

Our study has shown that the frequency of micronuclei in astrocytes is not increased by exposure to 50-Hz, 10-mT EMFs alone, but that co-exposure to 50-Hz, 10-mT EMFs increases the frequency of micronuclei induced by 5-FU. Some reports have shown that stress reactions increase or enhance the induction of micronuclei[37]. Our findings suggest two possible mechanisms for the increased genotoxicity induced by co-exposure to EMFs and 5-FU: stress reactions and tumor-promoting effects.

We have also studied the genotoxicity of strong static EMFs (0.15 to 11.75 T) on microbial systems, cultured cells, and animals[37-42]. The mutation rate in the TA98 *Salmonella* tester strain is decreased by co-exposure to furylfuramide and 11.75-T static EMFs and is increased by co-exposure to 5-nitroacenaphthene and 11.75-T static EMFs[38]. The frequency of micronuclei in Chinese hamster CHL/IU cells is decreased by co-exposure to mitomycin C and 4.7-T static EMFs[39]. The food and water consumption and body weight of mice are decreased after 48 hours' exposure to 4.7-T static EMFs[40]. The metallothionein content and lipid peroxidation in the livers of mice are enhanced by co-exposure to tetrachloride and 4.7-T static EMFs[41,42]. The frequency of micronuclei in mouse bone marrow cells in an *in vivo* study was increased by 3.0- and 4.7-T static EMFs alone[37]. Our previous studies have suggested that the effects of strong static EMFs may be enhanced or suppressed by the stress reaction or by changes in the cell cycle. Our findings suggest three possible mechanisms for the increased genotoxicity induced by co-exposure to cisplatin and EMFs: stress reactions, tumor-promoting effects, and active oxygen species. Extremely low-frequency magnetic fields (3- to 3,000-Hz) may be carcinogenic to humans (Group 2B) according to the International Agency for Research on Cancer[43]. Our study suggests that the threshold of genotoxicity induced by co-exposure to EMFs and 5-FU for 72 hours is between 7.5 and 10 mT. We have previously reported that the genotoxic activity of cisplatin is increased by co-exposure to 50-Hz, 7.5- or 10-mT EMFs and have suggested that the threshold of genotoxicity induced by co-exposure to EMFs and cisplatin for 72 hours is between 5 and 7.5 mT[6]. In homes and offices, the magnetic flux density may approach 2 mT from such devices as hair dryers, can openers, and induction-heating cookers[44]. The magnetic flux intensity of industrial equipment, such as arc welders and induction furnaces, may range from 0.1 to 60 mT[44]. Therefore, the possibility of exposure to several sources of EMFs at the milli-Tesla densities of our study is high. Our study suggests the importance of evaluating the risk of EMFs for humans.

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