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In vivo genotoxicity of high-intensity intermediate frequency magnetic fields in somatic cells and germ cells

Shin Ohtani[1], Akira Ushiyama[2,*], Keiji Wada[3], Yukihisa Suzuki[3] and Kenji Hattori[1]

[1]Department of Environmental Toxicology, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo, 204-8588, Japan
[2]Department of Environmental Health, National Institute of Public Health, 2-3-6 Minami, Wako, Saitama, 351-0197, Japan
[3]Graduate School of Science and Engineering, Tokyo Metropolitan University, 1-1 Minami-Osawa, Hachioji, Tokyo, 192-0397, Japan
*Corresponding author. Department of Environmental Health, National Institute of Public Health, 2-3-6 Minami, Wako, Saitama, 351-0197, Japan. Phone: +81-48-458-6254; Fax: +81-48-458-6270. E-mail: ushiyama.a.aa@niph.go.jp
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ABSTRACT

Intermediate frequency magnetic fields (IF-MFs) at ∼85 kHz are one of the components of wireless power transfer (WPT) systems. However, the available data needed for the assessment of the safety of organisms from IF-MF exposure are scarce. Thus, there is an imminent need to accumulate evidence-based assessment data. In particular, if humans are exposed to IF-MF due to an accident or trouble, they are at increased risk of being exposed to high-intensity IF-MF within a short period. The already existing exposure system was improved to a system that could intermittently expose animals at 3 s intervals. This system allows the exposure of a mouse to high-intensity IF-MF (frequency: 82.3 kHz; induced electric field: 87 V/m, which was 3.8 times the basic restriction level for occupational exposure in the ICNIRP guideline), while regulating the heat generated by the coil. *In vivo* genotoxicity after IF-MF exposure was assessed using micronucleus (MN) test, *Pig-a* assay, and *gpt* assay. The results of MN test and *Pig-a* assay in hematopoietic cells revealed that neither the reticulocytes nor the mature erythrocytes exhibited significant increases in the IF-MF-exposed group compared with that in the sham-exposed group. In germ cells, MN test and *gpt* assay outcomes showed that IF-MF exposure did not cause any genetic or chromosomal abnormality. Based on these data, there was no genotoxic effect of our set IF-MF exposure on somatic and germ cells. These findings can contribute to the widespread use of WPT systems as effective data of IF-MF safety assessment.

Keywords: intermediate frequency magnetic field (IF-MF); wireless power transfer system (WPT); genotoxicity; somatic cell; germ cell; rodent

INTRODUCTION

The development of a wireless power transfer (WPT) system using an intermediate frequency [1] magnetic field (IF-MF) at ∼85 kHz has promoted its future practical use [2]; the intermediate frequency ranges from 300 Hz to 10 MHz. When electric vehicles are charged, a high-intensity IF-MF is induced by the WPT system. Thus, the use of the IF-MF band is expected to become popular among the general public. However, there is an urgent need to accumulate science-based evidence because the safety assessment studies on IF-MF are currently insufficient. Only few studies have assessed the biological effects of IFs at ∼85 kHz in the WPT system. The accumulation of data on the biological effects of high-intensity IF-MF exposure is indispensable for the widespread utilization of WPT systems. Previous studies on the biological effect of intermediate frequency bands have been conducted at ∼20 kHz that was assigned to the induction heating (IH)-cooker hob. Researchers have assessed the safety of IF-MF exposure using animals, but no positive results have been reported [3–7]. Most studies have assessed the effects at intensities lower than the basic restriction for occupational exposure by the ICNIRP guidelines [8]. To rigorously assess the safety of WPT systems, we need to assess their biological effects using an exposure system that is capable of inducing higher intensity IF-MF exposures.

We considered the development of an exposure system for exposing mice to IF-MF. Humans may be exposed incidentally to the high-

intensity IF-MF generated by a sudden overcurrent before the safety device cuts off the exposure system in cases of equipment failure or accidents. In preparation for the widespread use of WPT systems, we need to assess the safety of high-intensity and short-term IF-MF exposure. The development of this exposure system is important to assure the quality of *in vivo* experiments. However, developing such a system requires specialized skills and knowledge as well as a lot of labor and expense. We developed the high-intensity and short-term IF-MF exposure system in stages to overcome the difficulty of this process. Our existing IF-MF exposure system [9] has been improved and refurbished [10]. To investigate the biological effects of small animals on the above exposure scenarios, we need an IF-MF exposure system that mimics such IF-MF exposure. The exposure system that was developed and improved in this study can expose mice to high-intensity IF-MF intermittently.

The International Agency for Research on Cancer, which is an intergovernmental agency that forms a part of the World Health Organization, has classified extremely low-frequency magnetic fields and radio frequency electromagnetic fields as 'possibly carcinogenic to humans' (Group 2B) [11, 12]. As the possibility of carcinogenesis has been noted for the frequency bands above and below the intermediate frequency band, carcinogenesis assessment of the intermediate frequency band is also needed. Thus, we assessed genotoxicity in mice using the IF-MF exposure system.

There is no established method for the assessment of the effects of IF-MF exposure on human health. Most IF-MF genotoxic assessment methods used in this study were selected according to the test guidelines of the Organization for Economic Cooperation and Development (OECD), which is used to assess the safety of chemicals [13]. However, effects of exposure to magnetic field, which is a form of physical energy, are substantially different from the effects of chemicals, which require consideration of intrinsic effects. Moreover, the extrinsic effects of IF-MF exposure should be considered. In particular, given that chromosomal abnormalities and genetic mutations in germ cells can affect generations, the genotoxicity should be assessed for germ as well as somatic cells. In this study, more effective methods along with the use of typical genotoxic test batteries associated with chemicals were used to assess genotoxicity.

The micronucleus (MN) test and *Pig-a* assay with flow cytometry were effective in genotoxic assessments. In fact, our previous study has shown the effectiveness of these methods with erythrocytes [14]. These tests enabled reproducible, high-throughput analysis using a small amount of blood [15–17] and allowed accurate and temporal analyses. Simultaneous analyses of reticulocytes and mature erythrocytes in animals over time can contribute to the acquisition of reproducible and accurate data. Moreover, compared with previous studies conducted using a similar approach, the negative and positive results could be used more effectively in this study. 'No effect' outcomes can reinforce the negative results of previous studies. Conversely, the 'affected' outcome allows the accumulation of data as a new finding in the safety assessment of the IF-MF exposure.

The MN test in germ cells is useful for initial screening to determine the genotoxicity in germ cells. We extended the assessment target to germ as well as somatic cells in this study because X-rays have been reported to cause genotoxicity [18, 19], and mammalian germ cells are highly radiosensitive to these rays [20–23]. Moreover, the testis is highly sensitive to X-irradiation. Thus, temporary infertility occurs when the human testis is exposed to a radiation dose of 0.1–2 Gy [24]. In fact, the accumulation of deoxyribonucleic acid (DNA) damaged by X-irradiation in germ cells is associated with (among others) spontaneous abortion and infertility [25]. Furthermore, it has been reported that the formation of mutations is a more likely cause of next generation genetic diseases [26]. As male reproductive organs are located outside the body, they may be directly affected by high-intensity IF-MF exposure. We decided to conduct genotoxic assessments in germ cells because there is limited data on the biological effects of high-intensity IF-MF bands on germ cells.

Another approach to assess genotoxicity is the *gpt* assay, which has been adopted and incorporated in the OECD guidelines as test No. 488, entitled 'Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays' [27]. The test guideline describes it as an *in vivo* assay to detect chemicals that may induce gene mutations in somatic and germ cells. This method has been reported to be effective in the assessment of mutagenicity of radiation or ultraviolet B (UVB), which are forms of physical energies [18, 19, 28]. Considering that IF-MF exposure is not as potent as these mutagens, the presence or absence of the effect is unclear and ambiguous when the biological effects of IF-MF are assessed. Therefore, a comprehensive genotoxic assessment using multiple assessment systems is required for the assessment of the biological effects of IF-MF exposure. We determined that the *gpt* assay was effective for assessing IF-MF exposure because genotoxicity could be assessed in somatic and germ cells.

In this study, we assessed the genotoxic effects of high-intensity IF-MF exposure using the MN test, *Pig-a* assay and *gpt* assays in murine somatic and germ cells, as part of the IF-MF safety assessment.

MATERIALS AND METHODS

Animals

The study was approved by the Committee for Animal Experiments at National Institute of Public Health (Approval Protocol No.30–009). We ensured that minimum pain and distress was inflicted to the animals in all procedures. All procedures complied with the principles of the 3Rs (or 4Rs).

Male C57BL/6JJmsSlc mice were used to perform the MN test and *Pig-a* assay, whereas male C57BL/6JJmsSlc-Tg (*gpt* delta) mice were used to conduct the *gpt* assay [29]. All study mice were purchased from Japan SLC Inc. (Shizuoka, Japan), maintained in a clean room at a room temperature of 23°C ± 1°C, humidity of ∼55% and a 12 h light/dark cycle (light on, 8:00–20:00 h). These mice were exposed to IF-MF at an age of 8 weeks.

IF-MF exposure, X-ray irradiation and *N*-ethyl *N*-nitrosourea (ENU)-administration

We modified the IF-MF exposure system used in previous studies [9] and constructed a new exposure system [10]. This system consisted of a power supply, inverter, capacitor and solenoid coil equipped with a chiller for cooling (Fig. 1A–C). A schematic of the IF-MF exposure system was provided by Matsubara *et al.* [10]. The system has an air-core coil to generate IF-MF. A mouse is placed at the center of the coil (Fig. 1C), which is composed of a hollow copper pipe. The copper pipes on the first and second layers have 16 and 6 × 2 turns,

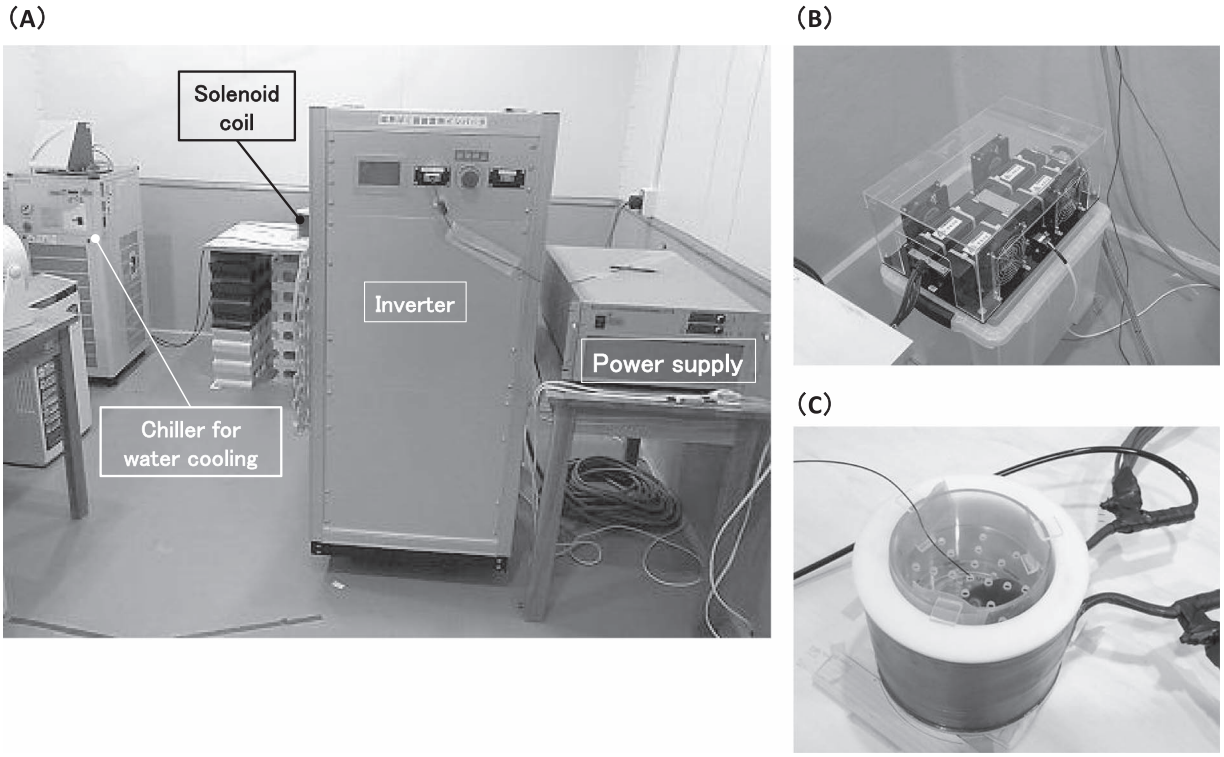

Fig. 1. Overview of the IF-MF exposure system (A), capacitor (B), and solenoid coil with the mouse-specific holder (C).

respectively. The temperature in the center of the coil during the IF-MF exposure was maintained at ~23°C by flowing water through the copper pipe. A new system could apply exposures of 3 s at 2-min intervals, generating the intermittent IF-MF exposure. The frequency of IF-MF was 82.3 kHz. Dosimetry was performed using the impedance method, as described in a previous study [30]. The exposure assessment was based on our previous study [14, 31]. The average induced electric field throughout the mouse body was estimated to be 87 V/m via numerical simulation. This was approximately 3.8 times of the basic restriction level for occupational exposure (22.9 V/m) indicated in the ICNIRP guideline [8]. The exposure duration lasted for 1 day or 10 consecutive days (Fig. 2), which was acquired 30 times per day. The total exposure time was 90 s per day.

Mice that were exposed to 3 Gy (0.642 Gy/min) of X-irradiation were considered as a positive control for the MN test ($n = 5$) and *Pig-a* assay ($n = 4$) of hematopoietic cells. Mice that were also exposed to 0.5 Gy (0.640 Gy/min) of X-irradiation were prepared for the germ cell MN test (n = 5) as a positive control. The mice were irradiated for 10 consecutive days using a Faxitron CP-160 X-ray apparatus (Faxitron X-Ray Corporation, Wheeling, IL, USA). ENU was dissolved in saline at an appropriate concentration and was administered intraperitoneally (50 mL/kg body weight) to mice once a day for 5 consecutive days ($n = 3$), and these mice were considered as a positive control for the *gpt* assay. Further, the liver, spleen, testis and bone marrow samples were collected 3 weeks after dosing.

Sampling points

The sampling points are summarized in Fig. 2. The final day of IF-MF exposure was determined to be day 0. Accordingly, the blood samples were collected from the tail vein before the IF-MF exposure and at days 0 (excluding for day 1 exposure), 2, 3, 6, 10 and 14 after the exposure for the MN test. Further, the samples were collected before the exposure and at 2, 7 and 14 days after the exposure for the *Pig-a* assay. The data obtained from reticulocytes and mature erythrocytes were compared among the IF-MF-exposed ($n = 6$), sham-exposed (n = 6) and X-irradiated ($n = 5$ or 4) groups in a pairwise manner.

The sampling point for the germ cell MN test and *gpt* assay was set at day 7 after the last IF-MF exposure. The results of the MN test were compared among the sham-exposed (n = 5), IF-MF-exposed (n = 5), X-irradiated (n = 5) and control (intact; n = 5) groups. The results of the *gpt* assay were compared between the sham- ($n = 6$) and IF-MF-exposed (n = 6) groups. The ENU-administrated (50 mg/kg × 5 days, $n = 3$) and intact (n = 3) groups were considered as positive and negative controls for the *gpt* assay, respectively.

MN test in hematopoietic cells

The MN test was conducted using the blood samples extracted at sampling points after 1-day and 10-days exposure, as described previously. Considering that detailed methods were described in our previous study [14], only brief descriptions are included below. Overall, 20 μl of blood was extracted from the tail vein of each mouse and was mixed with heparin solution. These samples were mixed with 1.3 mL of methanol and stored at −80°C for >3 days. Then, they were stained with the fluorescein isothiocyanate (FITC)-stained anti-mouse CD71 antibody, phycoerythrin (PE)-stained anti-mouse CD61 and propidium iodide that were enclosed in the Microflow kit (Litron Laboratories, Rochester, NY, USA). These were then washed and analyzed using CytoFLEX (Beckman Coulter, Fullerton, CA, USA). The

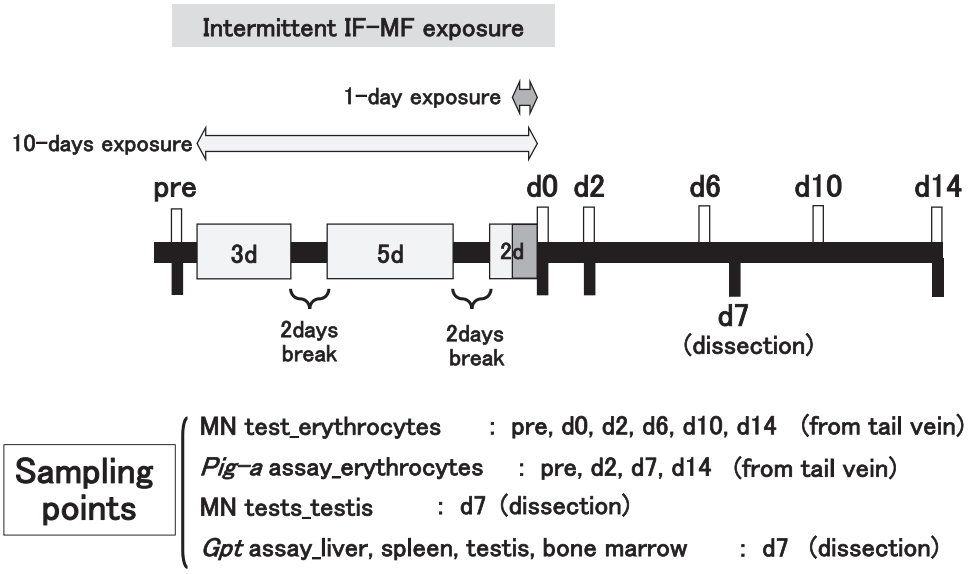

Fig. 2. Duration of IF-MF exposure and sampling points. In the MN test, the blood samples were extracted from mice before the exposure (pre) and at days 0, 2, 6, 10 and 14 after 1 day and 10 days of exposure. In the *Pig-a* assay, the blood was extracted before (pre) and at days 2, 7 and 14 after the exposure. The testis in the MN test and the liver, bone marrow, spleen and testis in the *gpt* assay were extracted from mice at day 7 after the last day of the 10-day exposure period.

acquired data were analyzed using CytExpert (Beckman Coulter), and the ratio of the MN frequency in erythrocytes was determined.

Pig-a assay in hematopoietic cells

The *Pig-a* assay was conducted using the blood samples extracted at sampling points after 1- day and 10-days exposure, as described previously. The methodology was also based on our previous study [14], as described below. Overall, 14 μL aliquots of the collected blood samples from mice were mixed with 10 μL of ethylenediaminetetraacetic acid (50 mM). These samples were reacted with FITC-labeled anti-mouse CD24 antibody (eBioscience, CA, USA), PE labeled anti-mouse CD71 antibody (eBioscience) and phycoerythrin-cyanin 5 (PC5) labeled anti-mouse TER-119 antibody (eBioscience). Further, they were washed and resuspended with phosphate buffered saline (PBS, Wako Pure Chemical Industries, Osaka, Japan) and were analyzed using CytoFLEX (Beckman Coulter). The acquired data were analyzed using CytExpert (Beckman Coulter).

MN test in germ cells

The MN test in germ cells was conducted using the testis samples extracted after 10-days exposure. The testis samples extracted at day 7 after exposure were fractionated by rubbing with two slides. These samples were filtered with a nylon mesh, washed with PBS and fixed in Farmer's fixative (3:1 ethanol:glacial acetic acid). The centrifuged samples were dropped on a Matsunami adhesive silane-coated slide glass (Matsunami Glass, Osaka, Japan). After adequate drying, they were stained with acridine orange solution (500 μg/mL) (Fujifilm Wako Pure Chemical, Osaka, Japan) in an incubation chamber for 10 min. Subsequently, they were washed using a 0.1 M phosphate buffer (pH = 6.4) solution, stained using 4′,6-diamidino-2-phenylindole (Sigma–Aldrich, St Louis, MO, USA) for 10 min, and observed microscopically. Images were acquired under an all-in-one fluorescence microscope (BZ-710; Keyence, Osaka, Japan). The plugin 'cell counter' of the software ImageJ/Fiji (National Institutes of Health, Bethesda, MD, USA) was used to distinguish spermatids, spermatocytes, and spermatogonia and count the number of micronucleated cells. All counts were performed in a single-blinded manner.

Gpt assay

The *gpt* assay was performed following the method described by Nohmi [29, 32]. Our methodology is described below. Briefly, DNA was extracted from the liver, spleen, bone marrow and testis of mice 7 days after IF-MF exposure. These tissues were processed using proteinase K, and the proteins in these solutions were removed via the extraction with phenol, chloroform and isoamyl alcohol (25:24:1). The genomic DNA was purified via ethanol precipitation. The lambda EG10 DNA was rescued in the form of phages using the *in vitro* packaging reaction with Transpack Packaging Extract (Agilent Technologies, Santa Clara, CA, USA), as per the following protocol. The Lambda EG10 phages were transfected into *Escherchia coli* YG6020 that expressed Cre-recombinase and generated a 6.4 kb plasmid, which carried the *gpt* and chloramphenicol acetyltransferase genes. Mutations in the *gpt* gene were selected using 6-thioguamine (6-TG). We compared the mutant frequency values in these organs between the sham- and IF-MF-exposed groups. The mutant frequency denotes the total number of colonies on the mutation-detection plate (M9 + Chloramphenicol (Cm) + 6-TG) multiplied by 10^6 and divided by the total number of colonies on the normal-detection plate (M9 + Cm). The ENU-administered group was used as the positive control, whereas the untreated group was used as the negative control.

Statistical analysis

The frequency of MN and *Pig-a* mutations in reticulocytes and mature erythrocytes was compared among the IF-MF-exposed ($n = 6$), sham-exposed (n = 6) and X-irradiated ($n = 5$ or 4) groups. The frequency of presence of micronuclei in germ cells were compared among the IF-MF-exposed (n = 5), sham-exposed (n = 5), X-irradiated (n = 5) and control (n = 5) groups. These comparisons were statistically analyzed via the Kruskal–Wallis tests using the JASP software (http://www.jasp-stats.org). If significant differences were observed, Dunn's test was performed as a post-hoc test. Significance levels were set at $P < 0.05$.

The mutant frequency in the *gpt* assay was compared between the sham- ($n = 6$) and IF-MF-exposed (n = 6) groups using Student's or Welch's *t*-tests when these groups yielded equal or unequal variances, respectively. Significance levels were set at $P < 0.05$.

RESULTS

Genotoxicity in hematopoietic cells

We analyzed the MN mutation frequency in reticulocytes and mature erythrocytes after 1-day or 10-days exposure. These results are summarized in Table 1. Three groups, namely sham-exposed, IF-MF-exposed and X-irradiated groups, were compared using the Kruskal–Wallis test. Significant differences were observed at days 2, 6 and 10 after exposure in reticulocytes and at days 10 and 14 in mature erythrocytes. The post-hoc Dunn's test was conducted at these sampling points. The results showed that after 1-day and 10-days exposure, no statistically significant differences in reticulocytes or mature erythrocytes were observed between the sham- and IF-MF-exposed groups at all sampling points. The mutation frequency of the X-irradiated group, which was used as the positive control, was statistically higher than that of other groups.

The *Pig-a* mutation frequency was also analyzed in reticulocytes and mature erythrocytes after 1-day and 10-days exposure. These results are presented in Table 2. As we did with the MN test, the three groups were also compared using the Kruskal–Wallis test. In reticulocytes, significant differences were observed at day 7. In mature erythrocytes, significant differences were observed at days 2, 7 and 14. In the post-hoc Dunn's test, no significant differences in reticulocytes and mature erythrocytes were noted between the IF-MF- and sham-exposed groups for all sampling points except day 2. At day 2 (after 1-day and 10-days exposures) in mature erythrocytes, the *Pig-a* mutation frequency of the IF-MF exposed group was lower than that of the sham-exposed group. The mutation frequency of the X-irradiated group (used as a positive control) showed high values at day 7 in reticulocytes and tended to increase at day 14 in mature erythrocytes.

Chromosomal aberrations in germ cells

To detect the chromosomal aberrations in germ cells, the MN frequency was estimated in spermatids or mixed germ cells. Following microscopic observations, these cells, which were placed on the slides, were classified using the ImageJ software into spermatids, spermatocytes, spermatogonia and sperm (Fig. 3). The minimum/maximum number of total mixed germ cells and total spermatids estimated in this study was 3800/12149 and 2054/5819, respectively. In spermatids, no significant differences were observed between the sham- and IF-MF-exposed groups after 10-days exposure (Fig. 4B). Similarly, no significant differences were noted between the sham- and IF-MF-exposed groups in mixed germ cells (Fig. 4A). In both cells, the MN frequency of the X-irradiated group as a positive control were significantly higher than that of other groups (sham-exposed, IF-MF-exposed and control groups).

Gpt assay in somatic and germ cells

Table 3 lists the mutant frequency data of the *gpt* assay in liver, spleen, bone marrow and testis. The results showed no statistically significant differences between the sham- and IF-MF-exposed groups. Positive (ENU-administrated) and negative (intact) control groups were prepared and compared with the values of the two exposure groups. The mutation frequencies of the IF-MF- and sham-exposed groups were smaller than that of the positive control group but were approximately the same as that in the negative control group.

DISCUSSION

The *in vivo* genotoxicity of IF-MF exposure was assessed as part of the safety assessment of IF-MF using appropriate methods, such as the MN test, *Pig-a* assay and *gpt* assay. We could expose the mice to IF-MF using the modified IF-MF exposure system at the maximum output generated at that time. According to numerical estimation (obtained from the simulation using the impedance method), IF-MF generated an average induced electric field of 87 V/m throughout the mouse body. This was 3.8 times higher than the basic restriction level for occupational exposure, as indicated in the ICNIRP guidelines (22.9 V/m). Our study was the first *in vivo* research to assess the genotoxicity of IF-MF at this intensity. We showed that the intermittent IF-MF exposure in our study did not induce genetic mutations or chromosomal abnormalities in somatic and germ cells. Our genotoxic assessment data can significantly contribute to the accumulation of data on the biological effects of IF-MFs at ∼85 kHz based on scientific evidence. We believe that these findings will be applied to practical use of the WPT system.

One of the objectives of this study was to assess the genotoxicity of high-intensity IF-MF to which living organisms are exposed under uncertain situations, such as accidents. In the event of an accidental overcurrent, we assumed that the safety device would take a few seconds to shut down. Furthermore, to allow for a margin of safety, the number of exposed shots was set to 30 times per day, and the duration of exposure was set to 1 day or 10 consecutive days. It is important to assess genotoxicity at high doses and clarify the threshold at which IF-MF shows genotoxicity. However, as mentioned in the Introduction, the construction of the exposure system has technical, financial and labor problems, and there are limits to the strength of the magnetic field that can be generated. The upper limit of this system was to generate an average induced electric field of 87 V/m throughout the mouse body. This value of induced electric field is 3.8 times the basic restriction for occupational exposure in the ICNIRP guidelines, and we believe it is sufficient to assess the safety of IF-MF exposure. On the other hand, we attempted to assess the short-term effects of IF-MF, but we could not assess the chronic effects of IF-MF. We believe that the assessment of chronic effects is a challenge for future research. In the future, we plan to develop an IF-MF exposure system that can increase the exposure intensity and allow long-term exposure.

This study is broadly divided into genotoxicity assessments of IF-MF exposure in somatic and germ cells. First, we assessed the

Table 1. Results of the MN test after 1 day and 10 days exposure. Following the Kruskal–Wallis test, statistical analyses among the sham-exposed, IF-MF-exposed and X-irradiated groups were conducted using the post-hoc Dunn's test (* and ** denote statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively). The positive control group was compared with the 1 day- and 10 day-exposed groups (S1d: 1 day sham-exposed, E1d: 1 day IF-MF-exposed, S2w: 10 days sham-exposed and E2w: 10 days IF-MF-exposed). Normochromatic erythrocytes (NCE) are mature erythrocytes that lack ribosomes.

		Reticulocytes (RETs)						Normochromatic erythrocytes (NCEs)					
		MN frequency (sum Ave.) [%]				±S.D.	Number of counted RETs	Number of MN RETs	MN frequency (sum Ave.) [%]			±S.D.	Number of counted NCEs	Number of MN NCEs
1-day exposure	**1-day Sham (n = 6)**													
	Pre	0.413				0.045	114 618	475	0.212			0.015	6 114 507	12 968
	d2	0.395				0.063	116 970	464	0.209			0.027	5 757 599	12 038
	d6	0.346				0.079	118 496	412	0.201			0.021	4 769 838	9623
	d10	0.410				0.046	117 442	483	0.202			0.026	5 388 982	10 894
	d14	0.379				0.054	119 193	453	0.203			0.018	6 319 678	12 832
	1-day IF-MF (n = 6)													
	Pre	0.404				0.080	110 827	450	0.209			0.019	5 970 379	12 491
	d2	0.376				0.062	118 524	447	0.199			0.017	5 136 407	10 237
	d6	0.390				0.063	118 884	466	0.198			0.016	4 493 463	8918
	d10	0.396				0.042	116 813	464	0.206			0.022	4 333 050	8933
	d14	0.353				0.066	121 070	429	0.201			0.008	5 497 042	11 086
10-days exposure	**10-days Sham (n = 6)**													
	Pre	0.402				0.050	112 565	454	0.213			0.020	5 850 897	12 486
	d0	0.410				0.038	116 240	479	0.214			0.016	6 947 814	14 898
	d2	0.383				0.031	115 461	444	0.212			0.014	6 253 523	13 271
	d6	0.369				0.068	118 653	439	0.198			0.013	4 649 399	9247
	d10	0.376				0.088	111 016	419	0.214			0.010	4 682 021	10 045
	d14	0.351				0.052	117 802	415	0.201			0.012	5 988 271	12 044
	10-days IF-MF (n = 6)													
	Pre	0.397				0.057	99 973	398	0.234			0.032	4 930 755	11 585
	d0	0.397				0.058	118 196	471	0.209			0.015	6 164 232	12 910
	d2	0.420				0.045	117 566	496	0.224			0.015	5 619 596	12 638
	d6	0.351				0.079	118 238	416	0.215			0.018	3 686 168	7943
	d10	0.429				0.057	118 173	509	0.218			0.019	4 111 847	8963
	d14	0.368				0.049	118 634	438	0.211			0.018	5 824 101	12 313
Positive control	**X-ray (3 Gy) (n = 5)**		S1d, E1d, S2w, E2w							S1d, E1d, S2w, E2w				
	Pre	0.451				0.068	96 706	438	0.237		*	0.027	3 052 324	7263
	d0	0.403				0.068	100 411	406	0.213			0.015	5 233 015	11 160
	d2	2.068	**	**	**	*	0.848	21 166	447	0.212			0.011	23 772 514	50 532
	d6	1.634	**	**	**	**	0.255	96 478	1603	0.213	*	*	*	0.019	7 673 781	16 412
	d10	0.805	**	**	**	*	0.167	98 417	799	0.271	**	**	**	** 0.048	1 429 734	3879
	d14	0.458		*	*	*	0.081	98 666	454	0.314	**	**	**	** 0.050	1 474 352	4645

* < 0.05 ** < 0.01.

Table 2. Results of the Pig-a assay after 1 day and 10 days of exposure. Following the Kruskal–Wallis test, statistical analyses among the sham-exposed, IF-MF-exposed and X-irradiated groups were conducted using the post-hoc Dunn's test (* and ** denote statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively). The positive control data quoted the values obtained in our previous study [14]. The positive control group was compared with the 1-day- and 10-day-exposed groups (S1d: 1-day sham-exposed, E1d: 1-day IF-MF-exposed, S2w: 10-days sham-exposed, and E2w: 10-days IF-MF-exposed). NCEs are mature erythrocytes that lack ribosomes.

		Reticulocytes (RETs)				Normochromatic erythrocytes (NCEs)			
		$CD24^-$RETs* 10^{-6} (sum Ave.)	±S.D.	Number of counted RETs	Number of $CD24^-$RETs	$CD24^-$NCEs* 10^{-6} (sum Ave.)	±S.D.	Number of counted NCEs	Number of $CD24^-$ NCEs
1-day exposure	**1-day Sham (n = 6)**								
	Pre	79.632	101.732	263 713	21	4.392	1.959	25 046 054	110
	d2	16.777	18.029	357 643	6	4.013	2.530	23 422 700	94
	d7	24.532	46.609	489 158	12	1.655	1.150	22 956 021	38
	d14	29.687	10.490	269 482	8	2.766	1.726	20 606 062	57
	1-day IF-MF (n = 6)								
	Pre	52.298	36.226	267 697	14	2.596	3.546	23 493 765	61
	d2	31.896	76.771	470 276	15	2.409*	4.417	24 076 984	58
	d7	14.321	54.808	488 800	7	1.675	1.676	23 284 701	39
	d14	56.086	79.960	392 257	22	1.869	2.294	21 936 310	41
10-days exposure	**10-days Sham (n = 6)**								
	Pre	32.621	67.553	275 900	9	2.280	1.830	23 680 493	54
	d2	30.175	79.602	364 541	11	4.047	2.814	27 425 602	111
	d7	24.287	22.550	411 739	10	4.415	4.402	22 648 625	100
	d14	92.586	68.554	302 423	28	3.224	1.421	21 402 272	69
	10-days IF-MF (n = 6)								
	Pre	25.273	24.012	276 976	7	1.963	2.115	22 927 547	45
	d2	30.183	26.974	364 442	11	0.930*	0.730	22 572 171	21
	d7	48.445	21.757	598 617	29	2.226	0.605	22 009 015	49
	d14	64.280	70.926	326 698	21	2.469	1.651	21 872 720	54
Positive control	**X-ray (3 Gy) (n = 4)**	S1d, E1d, S2w, E2w				S1d, E1d, S2w, E2w			
	Pre	11.97 * *	13.04	417 851	5	3.15	1.56	13 000 140	41
	d2	196.87	249.56	10 157	2	9.66 * **	5.42	12 841 955	124
	d7	175.26 ** ** *	90.61	462 091	81	9.73 ** *	8.32	11 406 036	111
	d14	21.60 *	41.03	833 223	18	10.65 * ** * *	6.79	12 768 486	136

* < 0.05 ** < 0.01.

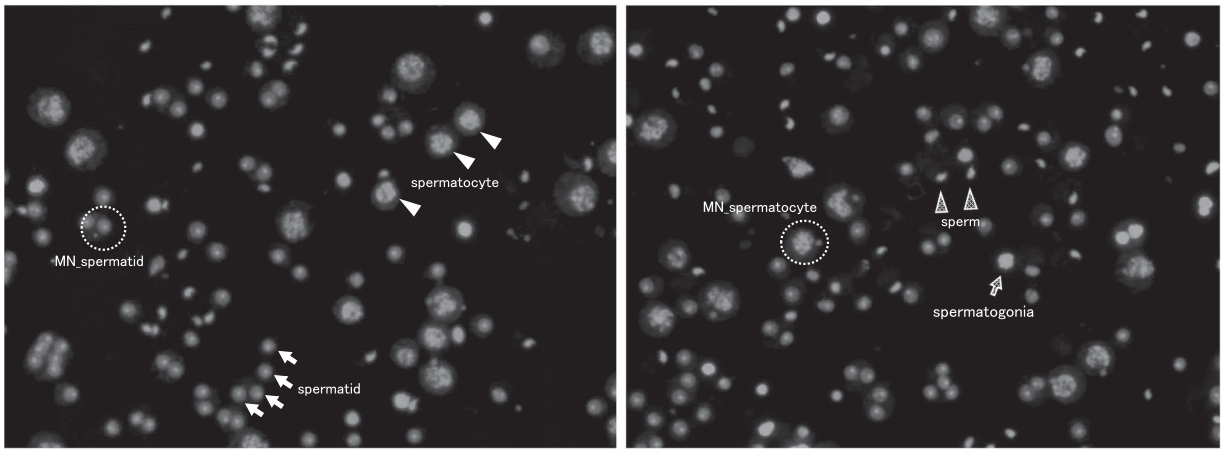

Fig. 3. Classification and identification of germ cells composed of micronuclei (or not). These images show germ cells in the testis. A typical MN-contained spermatid is indicated by a dotted circle, a typical spermatid is indicated by a filled arrow and a typical spermatocyte is indicated by a filled triangle, as shown in the image on the left. A typical MN test outcome suggested that the samples contained the following cells: spermatocytes indicated by a dotted circle, spermatogonia indicated by an arrow filled with dots, and a typical sperm indicated by a triangle filled with dots, as shown in the image on the right.

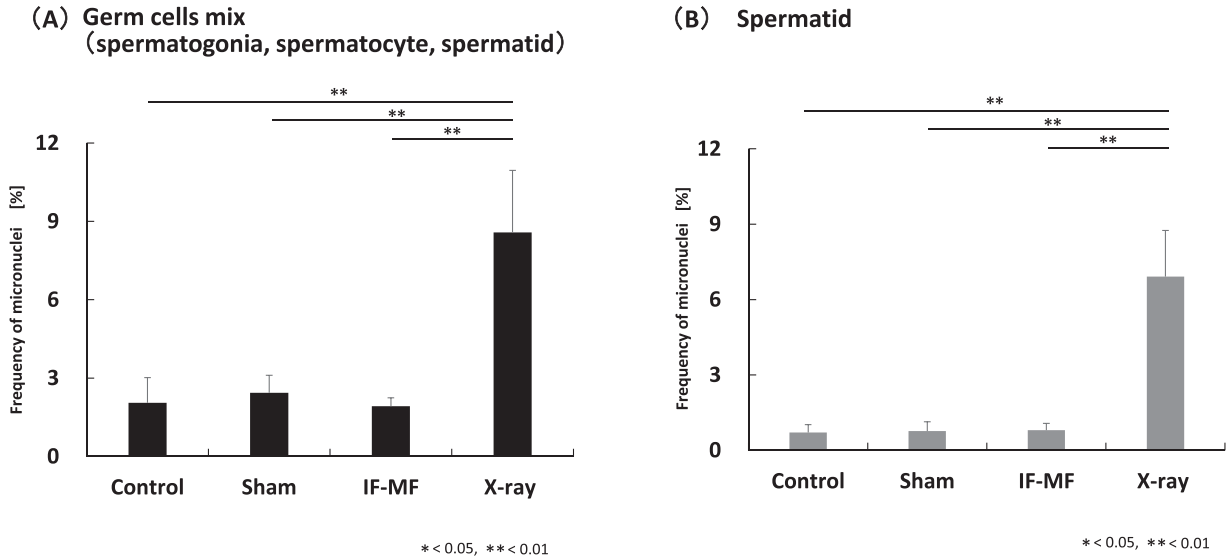

Fig. 4. MN mutation frequency in germ cells. (A) denotes mixed germ cells (spermatids, spermatocytes and spermatogonia), and (B) denotes only spermatids. Following the Kruskal–Wallis tests, the significant levels were analyzed using the post-hoc Dunn's test (* denotes $P < 0.05$ and ** denotes $P < 0.01$).

genotoxicity of IF-MF exposure in somatic cells. The results of the MN test after 1-day and 10-days IF-MF exposure showed that there were no significant differences in reticulocytes and mature erythrocytes at all sampling points. Moreover, these results showed that IF-MF exposure at these conditions did not cause major chromosomal defects or chromosomal abnormalities in somatic cells. A similar trend was observed in the *Pig* assay performed using reticulocytes and mature erythrocytes. Although significant differences were observed at day 2 after the final exposure in the IF-MF-exposed group, the *Pig-a* mutation frequencies of the IF-MF-exposed group were significantly lower than those of the sham-exposed group after 1-day and 10-days exposure. Therefore, these differences were not considered to affect the genotoxicity of IF-MF exposure. Considering that the *Pig-a* mutation frequency was assessed at 10^6, there was considerable variability. We believe that the variation was within the baseline of the *Pig-a* assay in mature erythrocytes.

To supplement the authenticity of the *Pig-a* assay results, the *gpt* assay was performed using other somatic cells of the liver, spleen and bone marrow after IF-MF exposure. When the mutagen reactivity is unclear in the assessment of the biological effects of IF-MFs, it is important to conduct a comprehensive assessment using multiple methods. Based on the results of the *Pig-a* and *gpt* assays, the IF-MF

Table 3. Results of the *gpt* assay after 10-days exposure in somatic and germ cells. Statistical analyses were conducted using the Student's or Welch's *t*-tests when these groups yielded equal or unequal variances, respectively (NC: negative control, PC: positive control, MF: mutant frequency, SD: standard deviation)

	Exposure method	Average MF ($\times 10^{-6}$)	± SD
Liver	**NC (n = 3)**	13.2	9.5
	PC (n = 3)	218.3	58.6
	Sham (n = 6)	33.9	9.7
	IF-MF (n = 6)	28.3	15.5
Bone marrow	**NC**	10.4	9.2
	PC	276.0	136.2
	Sham	24.1	26.0
	IF-MF	17.8	15.6
Spleen	**NC**	15.9	13.6
	PC	322.7	105.8
	Sham	23.7	14.6
	IF-MF	5.3	6.7
Testis	**NC**	10.4	9.2
	PC	61.0	27.6
	Sham	10.3	7.0
	IF-MF	19.6	17.3

exposure under the study conditions did not cause genetic mutations in somatic cells. In addition, combining the results of this study and our previous study, we reconfirmed that IF-MF exposure does not induce genotoxicity in somatic cells [14].

Subsequently, we assessed the genotoxicity of IF-MF exposure in germ cells. The sampling points had been considered before we performed the analysis. As MN formation can occur at any stage of spermatogenesis, an assessment time point should be selected during this process. Spermatogenesis requires 35 days, and all spermatogonia, spermatocyte and spermatid stages last for approximately 10 days [33]. We inferred that the best sampling timing was during spermatogenesis, before sperm was released from the testis. The sampling point was assumed to be approximately 20 days after the first IF-MF exposure, considering the nature of spermatogenesis and the duration of each stage. As shown in Fig. 2, specifically, we dissected mice at 7 days after the final IF-MF exposure, which was 21 days after the first IF-MF exposure.

We performed the MN test using spermatids and mixed cells containing cells from three stages, as previously explained. The reason for using only spermatids was to assess the effects on the subsequent generation, such as the occurrence of malformations induced by structural chromosomal abnormalities. Abnormal cells may be metabolized at the stage of spermatogonia and spermatocytes; therefore, assessment was performed only using spermatids, which is the stage immediately before the sperm. Alternatively, the assessment using mixed cells helps in understanding the effects of IF-MF exposure on the chromosomal structure in spermatogenesis. Analysis of mixed cells can reveal the degree of direct chromosomal damage caused by IF-MF exposure. No significant differences between the sham- and IF-MF-exposed groups were observed, indicating that the IF-MF exposure under the given conditions did not cause any chromosomal breaks and abnormalities.

The *gpt* assay was also performed to assess the genetic mutation caused by IF-MF exposure in germ cells. Spermatogonia are special cells that not only undergo mitosis to become spermatocytes but also have self-renewing ability as stem cells. A single or multiple mutation in mouse spermatogonia can affect the next generation because sperm stem cells exhibit long-term self-renewing replication activity [34]. This could further result in intergenerational effects [35]. The negative results of the *gpt* assay indicated that the mutant frequency was not increased via IF-MF exposure in spermatogenesis. These findings of the MN test and *gpt* assay in germ cells indicated that IF-MF exposure does not cause chromosomal aberrations or mutations that result in genetic-based disease in the subsequent generations.

The results of the MN test, *Pig-a* assay and *gpt* assay along with the use of the IF-MF exposure system showed that the genotoxic assessment of IF-MF exposure was negative. The average induced electric field generated by the exposure system in whole mouse body was 87 V/m, which was 3.8 times of the basic restriction for occupational exposure indicated in the ICNIRP guidelines (22.9 V/m). In other words, the IF-MF exposure under this condition did not cause genotoxicity in somatic and germ cells. As the results of this study were based on genotoxicity assessment of short-term IF-MF exposure, chronic effects were not considered. In future work, it will be important to develop an IF-MF exposure system with higher intensity and conduct genotoxic assessment of the chronic effects of IF-MF.

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CONFLICT OF INTEREST

The authors declare they have no conflicts of interest to report.

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PRESENTATION AT A CONFERENCE

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DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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