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Original Article

Development of an *in vivo* pain assessment method for exposure to intermediate-frequency magnetic fields

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ABSTRACT — Research data on the biological effects of intermediate-frequency magnetic fields (IF-MF) remain inadequate, and there are no protocols currently exists that can assess the biological effects of electromagnetic fields similar to those used with the OECD guidelines for chemicals. IF-MF <100 kHz have a dominant stimulatory effect, which has raised concerns about their effects on neurological disorders. The purpose of this study was to investigate methods for detecting pain in response to IF-MF exposure and to establish a standardized protocol for electromagnetic field pain assessment for use in various environments. The von Frey test, which can assess foot pain, was performed using the partial sciatic nerve ligation (PSL) model, which is a nerve hypersensitivity and allodynia model, together with IF-MF-exposed, sham-exposed, and no-treatment (control) groups. Significant changes were observed at all postoperative measurement points in the PSL group, whereas no significant differences were present among the other groups. In addition, gene expression analysis for four inflammation-related factors (P2rx4, Ccl2, Irf8, and Iba1) was performed using real-time quantitative PCR in the sciatic nerve on postoperative day 15 after exposure. The expression of these genes was significantly upregulated in the PSL group but was unchanged in the remaining three groups. These results confirm that IF-MF exposure (1 hr/day), which is 2.3 times higher than the basic restriction for occupational exposure according to the ICNIRP guidelines, does not cause pain and that these detection methods with positive controls are effective as pain assessment methods for IF-MF exposure.

Key words: Intermediate-frequency magnetic field exposure, Pain assessment, Partial sciatic nerve ligation model, von Frey test

INTRODUCTION

The use of electromagnetic fields has become an integral part of daily life with both the low-frequency range used in power lines and power supplies and in the radiofrequency electromagnetic fields used in cell phones and Wi-Fi, which have become widespread in ordinary homes as technology has improved in recent years. More recently, intermediate-frequency magnetic fields (IF-MFs) have been used in induction-heating cookers and are expected to be used in wireless power-transfer systems for vehicle charging. Although research has been conducted on biological effects in the low- and high-frequency bands, limited research has been performed on biological effects

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in the intermediate-frequency band. Recently, Lee *et al.* reported that most recent animal studies of IF-MFs have shown no effects on carcinogenesis, pregnancy, development, learning and memory behavior, or immunity (Lee *et al.*, 2023). We have also assessed the various biological effects of IF-MF via genotoxicity in mice (Ohtani *et al.*, 2023), immunotoxicity in developing rats (Ushiyama *et al.*, 2014), and comprehensive gene expression using the cerebrum and liver of mice (Ohtani *et al.*, 2019), as well as in studies using much higher levels of IF-MF, none of which showed any effects.

However, how the central and peripheral nervous systems are affected when living organisms are exposed to IF-MF in the 85-kHz band remains unclear as do the biological effects of long-term exposure. The effects of IF-MF on the organism are dominated by thermal effects >100 kHz and electrical stimulation <100 kHz (ICNIRP, 2010); thus, an 85-kHz band is dominated by electrical stimulation. Therefore, concerns have been raised regarding the effects of IF-MFs on nerve stimulation and neuropathy. There are no established methods are available for assessing the safety of electromagnetic fields, such as outlined in the OECD Test Guidelines for Chemicals and Other Substances. Several studies that have been conducted may have been of poor quality in terms of the study design itself and used facile electromagnetic field exposure systems. Consequently, research methods are required that can provide robust scientific evidence and that can be agreed upon internationally.

One of the most common methods used to assess pain is the mechanical stimulation test, which is a form of behavioral analysis. These include the von Frey test, a method of assessing pain by applying a filament to the sole of a rodent's foot and measuring the time and pressure required to withdraw the foot (Seltzer *et al.*, 1990). In addition, the dynamic plantar aesthesiometer (Ugo Basile SRL, VA, Italy), which raises the filament at a fixed rate of force increase, measures the time between contact of the filament with the animal's foot and its release and can reduce experimenter variability (Petrus *et al.*, 2007).

IF-MF is a nonionizing radiation and does not provide the strong damaging stimulus of ionizing radiation (e.g., X-rays, γ -rays), which can damage DNA; therefore, biological changes are minimal, and a positive control that exhibits clear changes and a sensitive detection method are required to assess pain in response to IF-MF exposure. The sciatic nerve is the thickest of the peripheral nerves and potentially the most sensitive to IF-MF. The partial sciatic nerve ligation (PSL) model has been reported to be a useful model of nerve hypersensitivity and allodynia (Seltzer *et al.*, 1990) and is an excellent positive control for chronic pain (Korah *et al.*, 2022) and was selected as such for this study. We chose real-time quantitative PCR (RT-qPCR) analysis of the sciatic nerve as a sensitive method to detect changes that could not be detected by behavioral analysis.

This is a pilot study to establish a pain assessment method for IF-MF exposure. We developed a protocol that considers the IF-MF exposure method and pain assessment, as well as the OECD guidelines for chemicals. In the behavioral analysis, the von Frey test for pain assessment was performed using the dynamic plantar aesthesiometer. For more detailed analysis, we performed gene expression analysis of macrophage-related inflammatory factors in the sciatic nerve. In addition, we established an IF-MF exposure method and procedure for reproducible and accurate exposure.

MATERIALS AND METHODS

Animal

The von Frey test was performed using inbred (BALB/ cCrSlc, C57BL/6NCrSlc), hybrid (CB6F1/Slc: BALB/ cCrSlc (female) × C57BL/6NCrSlc (male)) and outbred (Slc: ICR) strains to select the BALB/c strain with the lowest variation and the most stable values. All mice were purchased from SLC Inc. (Shizuoka, Japan), and male mice with less hormonal imbalance were selected. BALB/c mice were used to perform all experiments in this study. Mice were kept in a special shielded room with a room temperature of $23^{\circ}C \pm 1^{\circ}C$, humidity of ~55%, and a 12-hr light/dark cycle (light on: 8:00–20:00 hr). Mice were transferred to a separate shielded room during IF-MF exposure.

This study was approved by the Committee for Animal Experiments at our institute (Approval Protocol No. R4-003). All procedures ensured that minimal pain and distress was inflicted on the animals. All procedures adhered to the principles of the 3Rs.

Preparation of the PSL model

The PSL model was based on the method described by Seltzer *et al.* (1990). Briefly, BALB/c mice were subcutaneously injected with a ketamine–xylazine mixture (10 and 1 mg/kg, respectively), the left leg was shaved with clippers, and the sciatic nerve was exposed with a scalpel and scissors. The sciatic nerve was ligated up to 1/2–2/3 with a needle made of silk 6–0 thread (Natsume Co., Tokyo, Japan). After ligation, the muscle layer was sutured with 6–0 silk suture (Natsume Co.) and the skin was sutured with 4-0 silk suture, and mice were observed

In vivo pain assessment for IF-MF exposure



Fig. 1. IF-MF exposure system. (A) The IF-MF-exposed system containing the power supply, control computer, inverter (containing the capacitor), water-cooling device, and equipment for measuring current and temperature around coils. (B) An overview of the water-cooling circuit. The thermocamera, which is part of the temperature measurement device, was placed in the center of the diagram. (C) Simulation of the magnetic field density distribution at the coil center, fed by an electric current of 30 A. (D) and (E) show the solenoid coil and the acrylic mouse holder.

until the anesthesia wore off. In the von Frey test, approximately 85% of mice that had received the PSL operation showed symptoms within 14 days (data not shown), indicating that they could be adequately used as positive controls for this study.

IF-MF exposure system

We exposed mice to the IF-MF intensity as used in previously developed IF-MF exposure system (Wada *et al.*, 2016). The system (Fig. 1A) comprises a power supply, an inverter (containing capacitor), a control PC, a solenoid coil, and a water-cooling device and circuit (Fig. 1B). The solenoid coil (Fig. 1C) was made of waterflowable copper tubing, which comprises two concentric layers, with each layer having 12 turns. The impressed magnetic flux density distribution produced by the coil was calculated using commercial computational electromagnetic software (CST Studio Suite, 2023) (Fig. 1D). At the coil center, the effective value of the impressed magnetic flux density was approximately 6.1 mT. IF-MF exposure was performed by placing the mouse in a special acrylic resin holder (Fig. 1E) and placing the holder inside the coil. The exposure period was 1 day (1 hr/ day) and the exposure frequency was 85 kHz. The electric field intensity induced in the entire body of the exposed mouse caused by the impressed magnetic flux density was calculated using an in-house program based on the three-dimensional impedance method (Orcutt and Gandhi, 1988).

A water-cooling circuit was incorporated to control the heat generated by the coils. This allowed the IF-MF exposure to be performed while reducing the thermal stress from the coil. Using the measurement device shown in Fig. 2A, the output current of 30 A (rms) was measured at 1-min intervals during IF-MF exposure. Simultaneously, the temperature at each of the five points around the coil (Tcoil 1: water entry path to the coil; Tcoil 2: top of the coil; Tcoil 3: inside the coil; Tcoil 4: bottom of the coil; and Tcoil 5: water exit path from the coil) that occurred during exposure was measured at 1-min intervals using a thermal camera, and the current waveform of each was also recorded.

The temperature in the coils was controlled to maintain the optimal rearing temperature by adjusting the air-



Fig. 2. (A) Measurement of current (RMS) values during IF-MF exposure, which is enclosed in the white dotted circle. The five measurement points (Tcoil 1–5) around the coil are shown in the figure. (B) An example comparing a realistic posture with a posed mouse model mimicking it. (C) Temperatures near the center of the coil were measured with a fiber optic thermometer. The top figure shows the installation status of the thermometer, and the bottom figure shows the results.

conditioning temperature and the water temperature setting of the water-cooling device. The temperature inside the holder during IF-MF irradiation was measured using a fiber thermometer (Fig. 2C). The ideal temperature in the coil is the optimal rearing temperature for mice $(21^{\circ}C-25^{\circ}C)$. Implementing these measures created an environment that was stress-free for the mice.

In addition to these arrangements for the exposure environment, a written procedure was developed to ensure that the IF-MF exposure was performed accurately.

Behavioral assessment

The *in vivo* pain assessment in this study began with the mechanical stimulation test. The von Frey test is a commonly used method for testing peripheral pain in the foot. This test assesses pain perception by stimulating the animal's foot with a filament and quantifying the time taken for foot withdrawal and pressure on the plantar surface. The dynamic planter aesthesiometer (Ugo Basile SRL) (Fig. 3A) was used to minimize the quantification error. The rate of pressure can be adjusted (1.0 g/sec in this study), and the measurement time between the application of pressure to the animal and withdrawal of the foot can be measured. Six months of training was used to familiarize animals with the dynamic planter aesthesiometer, and efforts were made to minimize the variability caused by use of the unit by different operators. Before all experiments, mice were allowed to acclimatize to the experimental environment for at least 10 min. The von Frey test was performed before exposure (or surgery) and 1, 5, 9, and 14 days after exposure (or surgery) to compare and assess leg withdrawal time and planter loading in the IF-MF-exposed, sham-exposed, PSL (positive control), and control groups.

Several strains of mice were prepared as described earlier, but the BALB/c strain was selected because of its high reproducibility and low error due to the number of days. The starting age of the experiment was 12 weeks, and the experiments were conducted with male mice, which do not require consideration of effects such as hormonal balance.

Sampling

The day of single exposure (1 hr/day) to IF-MF was designated as "day 0." On day 15 after IF-MF exposure, the sciatic nerve of the IF-MF– and sham-exposed groups was removed. The PSL group was similarly dissected



Fig. 3. (A) This diagram provides an overview of the dynamic plantar aesthesiometer. (B) Results of the von Frey test for leg withdrawal times at preexposure and 1, 5, 9, and 14 days postexposure between the IF-MF–exposed, sham-exposed, PSL, and control groups. (C) Comparisons in filament forces were made in the same way as in (B). The Kruskal–Wallis test was performed among the four groups, followed by the Dunn's test as a post hoc test (n = 5, P value: *< 0.05, **< 0.01). The asterisks of the PSL group in (B) and (C) indicate the comparison from top to bottom with the IF-MF-exposed, sham- exposed, and control groups.</p>

on day 15 after surgery, with the day of surgery designated as "day 0." All dissections were performed under anesthesia after exsanguination. Mice were anesthetized intraperitoneally (or subcutaneously) with a ketamine–xylazine mixture (10 and 1 mg/kg, respectively). The excised sciatic nerves were stored in RNA Protect reagent (Qiagen, CA, USA) at 4°C for 1–3 days and then at -80° C.

Molecular biology assessment

Total RNA from the sciatic nerve was extracted using RNeasy Mini Kits (Qiagen, Hilden, Germany), and cDNA was synthesized by reverse transcription using High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific, MA, USA). RT-qPCR was performed with Fast SYBR Green Master Mix (Thermo Fisher Scientific). Primers were designed within the amplified fragment length of 150-bp using Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm). Primer sequences were for genes encoding purinergic receptor P2X, ligand-gated ion channel 4 (*P2rx4*) forward: 5'- AGCTGCTCATCCTGGCTTAC-3', reverse: 5'-ACCACAGAGTCCGTTTCCTG-3'; C-C motif chemokine ligand 2 (Ccl2) forward: 5'- TCCCAAT-GAGTA GGCTGGAG-3', reverse: 5'-TCTGGACC-CATTCCTTCTTG-3'; interferon regulatory factor 8 (Irf8) forward: 5'- GCGAAGTTCCTGAGATGGAG-3', reverse: 5'-TGGGCTCCTCTTGGTCATAC-3'; induction of brown adipocytes 1 (Iba1) forward: 5'- CCGAG-GAGACGTTCAGCTAC-3', reverse: 5'-ACCAGTTG-GCCTCTTG TGTT-3'; and glyceraldehyde 3-phosphate dehydrogenase Gapdh forward: 5'-GAAGGTCGGT-GTGAACGG AT-3', reverse 5'-TGAGGTCAATGAA-GGGGTCG-3' (Eurofins Genomics Inc., Tokyo, Japan). RT-qPCR was performed at 95°C and 58°C for 45 cycles in a CFX Connect real-time system (Bio-Rad Laboratories, Hercules, CA, USA). The Pfaffl analysis method was used to analyze data, considering the reaction efficiencies.

Statistical analysis

All von Frey test and RT-qPCR data were assessed via nonparametric analysis of variance (ANOVA) and Kruskal–Wallis test. Dunn's test was performed as a post hoc test after ANOVA. The number of subjects required for the von Frey test was calculated using the power analysis software G*power3.1.9.7 (Faul *et al.*, 2009; Faul *et al.*, 2007). The effect size was determined from the PSL and control group mean (0.647 and 0.550, respectively) and standard deviation (1.39 and 3.20, respectively) of the leg withdrawal time in the von Frey test on day 9 day after PSL surgery and was calculated to be 3.014. Assuming a two-tailed test for the population correlation coefficient with a correlation coefficient of 3.014, a significance level of 5%, and a power of 95%, the required sample size was calculated to be five cases.

RESULTS AND DISCUSSION

Establishment of an IF-MF exposure system and development of an exposure environment

Herein, we investigated the construction of an IF-MF–exposure apparatus, assessment of IF-MF exposure, measurement of current values and waveforms during IF-MF exposure, temperature control during IF-MF exposure, IF-MF exposure procedures, and safety of IF-MF exposure. These points are essential for the development of future standard protocols and for conducting *in vivo* experiments, and the developed procedures should be included in the standard protocol.

To quantify IF-MF-exposure dosimetry, whole-body average and 99th percentile values of the electric field intensity induced in the entire body of an exposed mouse were numerically evaluated. The whole-body average value is the mean of all data inside the mouse body while the 99th percentile value excludes the largest 1% of the data to describe the highest value, as recommended by the ICNIRP guideline (ICNIRP, 2010) to avoid anomalous data from staircase approximation (Dawson et al., 2001). The simulation was conducted on a heterogeneous mouse model with adjustable postures, mimicking realistic postures observed in randomly captured photograms from a video recorded during an exposure experiment. Fig. 2B illustrates one of the six mouse models used. In our exposure system, the average whole-body electric field intensity for mice in six postures was 20 V/m and the 99th percentile value was 53 V/m. These values were approximately 0.9- and 2.3-times higher than the basic restriction for occupational exposure of 23V/m by the ICNIRP guideline. In this study, pain assessment was performed on mice exposed to high-intensity IF-MF (1 hr/day).

When exposing organisms to electromagnetic fields, accurate and reproducible IF-MF exposure is ideal. In this study, a new measurement device that could measure current values (RMS) and temperature around the coil was added to perform accurate IF-MF exposure with higher reproducibility than conventional exposure devices (Wada et al., 2016). Current values (RMS) and waveforms during IF-MF exposure, as well as temperatures at five locations around the solenoid coil (Tcoil 1-5), were detected using a thermal camera, and data were recorded (Fig. 2A). If abnormal temperatures were detected, equipment abnormalities and failures could be quickly detected. This is important to ensure safety during IF-MF exposure. Furthermore, to control changes in temperature around the coil, we attempted to keep the mice as healthy as possible during IF-MF exposure by maintaining the temperature inside the coil at the optimal temperature for mouse breeding. Using a water-cooling circuit, the temperature of the area inside the coil could be controlled to maintain the optimal temperature for rodents. The temperature inside the coil was measured using a fiber optic thermometer during IF-MF exposure (Fig. 2C). We confirmed that the optimal temperature $(21^{\circ}C-25^{\circ}C)$ for keeping mice can be maintained when the water flow was set at 11°C and the air conditioning was set at 22°C (Fig. 2C). The temperature inside the solenoid coil area was regulated by the water-cooling device and the air conditioner.

Operator safety must also be considered when performing high-power IF-MF exposures. First, a welldesigned IF-MF exposure protocol must be prepared to prevent operator errors. By performing IF-MF exposure operations according to the prepared procedures, we could avoid incorrect or irregular operations as much as possible and ensure safety during equipment operation.

Effectiveness of the pain assessment methods

The von Frey test is an excellent behavioral analysis method that allows direct quantification of the tactile sensation of the mouse paw. Herein, a dynamic planter aesthesiometer was used to reduce interoperator variability. We performed von Frey tests to compare foot withdrawal time and filament pressure among the control (negative control), sham-exposed, IF-MF-exposed, and PSL (positive control) groups. Significant decreases in leg withdrawal time and filament pressure were observed from the first day after surgery in the PSL group and were followed by a more pronounced trend; no significant differences were present between the IF-MF-exposed, shamexposed, and control groups (Fig. 3B, 3C). These results implied that a single (1 hr/day) IF-MF exposure had no effect on pain and that the change in value in the PSL group was significant and can be effectively used as a positive control. If repeated IF-MF exposure are required in the future or if the effects of long-term IF-MF exposure are assessed, the PSL model could also be used as a pos-



Fig. 4. Results of expression analysis using RT-qPCR for the sciatic nerve. Comparisons were made between the control, IF-MF– exposed, sham-exposed, and PSL groups. "Sh" and "IF" show the sham-exposed and IF-MF–exposed groups, respectively. Target genes were *P2rx4*, *Ccl2*, *Irf8*, and *Iba1*. For statistical analysis, the Kruskal–Wallis test was performed among the four groups, followed by the Dunn's test as a post hoc test (n = 5, P value: *<0.05, **<0.01).</p>

itive control for the assessment of chronic pain (Korah et al., 2022).

The von Frey test alone, which assesses pain, may not be sufficient to detect pain symptoms. Therefore, alterations in pain sensitivity were evaluated using a completely different method than behavioral analysis to confirm the results. We performed expression analysis using RTqPCR on the sciatic nerve of mice after IF-MF exposure. Macrophages play an important role in the regulation of inflammation (Domoto et al., 2021), and we consequently focused on four macrophage-associated inflammatory factors (P2RX4, CCL2, IRF8, and IBA1). P2RX4 is an ATP receptor, and the gene encoding this is one of the causative genes for increased allodynia (Jakobsson, 2010; Tsuda et al., 2003). CCL2, also known as monocyte chemoattractant protein 1 (MCP1), is an inflammatory chemokine that is upregulated in Alzheimer's disease and experimental autoimmune encephalomyelitis. (Conductier et al., 2010; Hickman and El Khoury, 2010; Ransohoff et al., 1993). IRF8 is a transcription factor and an important regulator of reactive microglia and is also involved in the enhancement of chronic pain (Masuda et al., 2012). IBA1 is a representative microglial and macrophage marker that can discriminate inflammatory responses from its posterior activity (Imai et al., 1996). The method for determining the presence or absence of pain using changes in the expression of inflammation-related genes as an indicator is very effective. The expression of *P2rx4*, *Ccl2*, *Irf8*, and *Iba1* was analyzed among the IF-MF-exposed, sham-exposed, PSL-exposed, and control groups. All four genes were significantly more expressed in the PSL group in the sciatic nerve on day 15 after exposure or surgery, with no significant differences among the control, IF-MF exposed, and sham-exposed groups (Fig. 4). These results indicated that no inflammation occurred in the IF-MF-exposed group. In the PSL group, all genes were significantly upregulated, indicating that RT-PCR is also effective for pain assessment.

Summary and further suggestions

As this was a pilot study to establish a pain assessment method for IF-MF exposure, exposure was first conducted with a 1-day exposure (1 hr/day). The results showed that no change was found in the IF-MF exposure group, but a significance effect was detected in the positive control, indicating that pain assessment based on the method used in this study is feasible. The 1-hr IF-MF exposure did not show a significant effect of IF-MF exposure on pain, but the effects of repeated exposure need to be evaluated for future understanding of the detailed effects of IF-MF. As described in the OECD guidelines for repeated oral toxicity study TG407 (OECD Guideline, 2008) and inhalation toxicity study TG412 (OECD Guideline, 2018), pain assessment after repeated exposure to IF-MF

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Investigation of in vivo pain assessment methods for IF-MF exposure. (schematic diagram)

Fig. 5. Schematic of the in vivo pain assessment methods for IF-MF exposure.

for 28 days is needed to assess subacute pain. Therefore, we are currently assessing pain in mice exposed to IF-MF for 28 days using the method established in this study. We are also conducting a histological analysis method to determine the presence or absence of inflammation by observing cellular infiltration using hematoxylin and eosin staining on tissue sections of the sciatic nerve and an enzyme-linked immunosorbent assay to assess the levels of C-reactive protein (CRP), a marker of inflammatory response, using plasma from each individual animal to understand the degree of inflammation. These methods, including behavioral and molecular biological analyses, are commonly used analytic tools in various fields, and we consider that researchers in various fields can propose appropriate protocols for assessing pain; we are also considering developing test methods that can be applied to any experimental setting. Ultimately, we plan to develop a standard protocol for in vivo pain assessment methods for IF-MF exposure.

The method of IF-MF exposure and in vivo pain assessment are summarized in Fig. 5 as a schematic. We propose a process of in vivo pain assessment for IF-MF exposure. The first step is to select the animal species. In this study, BALB/c mice were used, but rats, which have advantages in terms of ease of manipulation for behavioral analysis, could also be considered. Next, the exposure system must be constructed, the exposure environment prepared, and the exposure assessment performed, and people with relevant engineering expertise and technology skills are needed to support this work. Next, clear positive controls are necessary. In this study, the PSL model was used; at this point, it is recommended to determine the sample size required for behavioral analysis. Next, a method of pain assessment must be selected. For our evaluation, we combined behavioral analysis with molecular biological analysis (expression analysis). Depending on the experimental setting and implementation, combinations with other methods may also be chosen. The data obtained were analyzed using nonparametric statistics.

Finally, a series of evaluation experiments can be undertaken.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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