Original Article

Evaluation of the effects of power-frequency magnetic fields on the electrical activity of cardiomyocytes differentiated from human induced pluripotent stem cells

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ABSTRACT — Although cardiac activity is known to differ between species in many respects, most evaluations of the cardiac effects of low-frequency electric and magnetic fields, which have a stimulant effect on electrically activated cells, have been performed in non-human experimental animals and cells, and the effects in humans have been assessed using theoretical models. In recent years, it has been verified that human cardiomyocytes differentiated from human induced pluripotent stem cells (hiPS-CM) are useful for evaluating human responses to various cardioactive compounds. In this study, we applied hiPS-CMs for the first time to evaluate the human cardiac effects of power-frequency magnetic fields (MFs). After preparation of hiPS-CMs, we subjected a hiPS-CM monolayer formed on a multi-electrode array to short-term exposure to a 50 Hz MF at 400 mT with recording of the extracellular field potentials. The field potential duration of the hiPS-CMs did not differ significantly pre- and post-exposure, indicating that under these conditions, exposure to a 50 Hz MF at 400 mT does not affect the electrical activity of hiPS-CMs.

Key words: Power-frequency magnetic field, Cardiomyocyte, Human induced pluripotent stem cell, Extracellular field potential

INTRODUCTION

Exposure to time-varying and sufficiently strong electric and magnetic fields (EMFs) is known to have a stimulant or thermal effect on human tissues. Sensitivity to the two effects differs depending on the frequency of the EMF. EMFs < 100 kHz have a predominantly stimulant effect. In the current international guidelines/standards for protecting the human body from low-frequency (< 100 kHz) EMF exposure (The Institute of Electrical and Electronics Engineers, 2002; International Commission on Non-Ionizing Radiation Protection, 2010), threshold values that stimulate neurons in the central and peripheral nervous systems are used as the basis for restrictions because these are the cells with the highest sensitivity to EMFs (Reilly, 1998, 2002).

Muscle cells are also electrically activated cells. In particular, continuous rhythmic depolarization and repolarization of cardiomyocytes (CMs) is required for beat-

ing of the heart. Cardiac hazards, such as the threshold value for ventricular fibrillation, have been evaluated by magnetic or electrical stimulation of experimental animals (Elmas *et al.*, 2012; Fozzard and Schoenberg, 1972; Mouchawar *et al.*, 1989), and data from animal studies have been applied to humans after adjusting for body size (Geddes *et al.*, 1973). However, theoretical models are limited in their ability to predict human responses, as it is known that many aspects of fundamental cardiac activity, such as beating rate and electrocardiogram results (Boukens *et al.*, 2014; Denning *et al.*, 2016), differ between species. Although some human studies have been reported, the strength of EMF exposure was sufficiently low to be safe (Cook *et al.*, 1992; Korpinen *et al.*, 1993; Graham *et al.*, 1994; Korpinen and Partanen, 1994), and limited experimental data are available for direct evaluation of human effects. In Environmental Health Criteria 238 (World Health Organization, 2007) published by the World Health Organization, it was concluded that hazard-

ous cardiovascular effects associated with low-frequency EMFs are unlikely to occur at the exposure levels commonly encountered environmentally or occupationally; furthermore, the current international guidelines/standards based on nervous system sensitivity prevent cardiac hazard from electric stimulation, including that induced by exposure to low-frequency EMFs. However, because insufficient accumulation of knowledge regarding biological effects might reduce the reliability of risk assessment, and the effect on CMs is potentially life-threatening, it would be valuable to directly evaluate the responsiveness of human CMs to stimulant effects of low-frequency EMFs.

Human induced pluripotent stem (hiPS) cells, which are generated by reprogramming somatic cells using a particular set of transcription factors, have the ability to differentiate into multiple cell lineages, including CMs (Takahashi *et al.*, 2007; Yu *et al.*, 2007). CMs derived from hiPS cells (hiPS-CMs) are artificially generated source of human tissue, and their potential for use in medical and pharmacological applications has been intensively examined (Funakoshi *et al.*, 2016; Kawamura *et al.*, 2012; Khan *et al.*, 2013; Sirenko *et al.*, 2013). In the pharmacological field, hiPS-CMs have been confirmed to show appropriate responsiveness to known cardioactive compounds (Sirenko *et al.*, 2013), and hiPS-CMs are expected to contribute to efficient screening of candidate drugs (Khan *et al.*, 2013). HiPS-CMs are becoming a powerful tool to evaluate human responses. In this study, we applied hiPS-CMs for the first time to evaluate the biological effects of EMFs. After preparation of human CMs differentiated from hiPS cells, we subjected the hiPS-CMs to short-term exposure to a 50 Hz magnetic field (MF) at 400 mT and evaluated the effects by comparing the pre- and post-exposure extracellular field potentials of the hiPS-CMs.

MATERIALS AND METHODS

Cell culture

The hiPS cell line (201B7) (Takahashi *et al.*, 2007) was provided by the RIKEN BRC through the Project for Realization of Regenerative Medicine and the National Bio-Resource Project of the MEXT, Japan. The undifferentiated hiPS cells were maintained on a mitotically inactivated SNL76/7 feeder layer in Primate ES Cell Medium (ReproCELL, Kanagawa, Japan) supplemented with 10 ng/mL basic fibroblast growth factor (Wako, Tokyo, Japan). The culture medium was changed daily and the cells were passaged every 5 days.

Embryoid body formation and cardiomyogenic differentiation

The iPS cell colonies were detached with dissociation solution for human ES/iPS cells (ReproCELL). To induce formation of embryoid bodies (EBs), the colonies were transferred to plastic Petri dishes and cultured in α MEM (Gibco, Grand Island, NY, USA) supplemented with 20% Knockout Serum Replacement (KSR; Gibco) and 0.1 mM β -mercaptoethanol (Gibco). Wnt-3a (R&D Systems, Minneapolis, MN, USA) was added (100 ng/mL) to the medium from days 1 to 3 (Tran *et al.*, 2009; Yazawa *et al.*, 2011). On day 6, the EBs were plated into gelatin-coated 35-mm culture dishes in α MEM supplemented with 5% fetal bovine serum (FBS; Gibco), 0.1 mM β -mercaptoethanol, 100 units/mL penicillin and 100 μ g/mL streptomycin. Subsequently, 50% of the medium was replaced every 3 or 4 days. Beating EBs were mechanically isolated under a microscope (Eclipse TS100, Nikon, Tokyo, Japan), and trypsinized to prepare cell suspensions of cardiomyocytes.

Immunofluorescence

Cell suspensions of hiPS-CMs were reseeded on the gelatin-coated interior of a handmade polydimethylsiloxane (PDMS) O-ring (inner diameter: 8 mm) adhered to a chamber slide. The cardiomyocyte monolayer that formed after more than a week of culture was fixed using 4% paraformaldehyde for 30 min at 4°C and permeabilized using 0.2% Triton X-100 for 15 min at room temperature. Then the cells were blocked with 10% FBS followed by incubation with primary antibodies, such as mouse IgG1 monoclonal anti- α actinin (1:400 dilution; Sigma-Aldrich, Bellefonte, PA, USA), mouse IgG1 monoclonal anti-MHC (1:100; Abcam, Cambridge, UK), mouse IgG2b monoclonal anti-Troponin T (1:100; Abcam), mouse IgG2a monoclonal anti-GATA4 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat polyclonal anti-Nkx2.5 (1:50; Santa Cruz Biotechnology), and rabbit polyclonal anti-ANP (1:50; Santa Cruz Biotechnology). The secondary antibodies were all purchased from Molecular Probes (Eugene, OR, USA): Alexa Fluor 488-conjugated goat anti-mouse IgG1 (1:500) and chicken anti-mouse IgG (1:500) and Alexa Fluor 568-conjugated goat anti-mouse IgG2a (1:500), donkey anti-goat IgG (1:500), and goat anti-rabbit IgG (1:200). Stained samples were observed by fluorescent microscopy (Eclipse Ti-U, Nikon).

Multi-electrode array (MEA) recording

Extracellular field potential (FP) recordings of the

hiPS-CMs were performed using the MEA system (MED64; Alpha MED Scientific, Osaka, Japan) and the accompanying software (Mobius QT). For drug loading tests, a hiPS-CMs monolayer was formed on an electrode array positioned in the center of the probe (MED-P515A; Alpha MED Scientific) using a PDMS O-ring as described above. The O-ring was removed several days later. ReproCardio2 Assay Medium (Repro CELL) was used as a recording medium. For testing of MF exposure, cardiomyocyte monolayers were formed independently on two areas of an electrode array in a probe (MED-P5003A). To provide higher exposure of hiPS-CMs to induced electric fields, a probe with electrode areas far from the center was used. Data were analyzed using the Mobius QT software.

Drug loading test

After the medium on the MEA probe was exchanged for recording medium, the probe was incubated in the environmental chamber of the microscope at 37°C for 90 min. Then, the FPs were recorded for 5 min. Subsequently, a stock solution of the drug to be tested was added to the medium at 0.1% dilution and the FPs were recorded for 5 min following a 10-min incubation. The drugs tested were E-4031, verapamil, and aspirin (all from Sigma-Aldrich).

MF Exposure

For MF exposure, we used a previously reported system (Nakasono *et al.*, 2003), with different settings. The system comprised a pair of saddle-shaped coils and a window frame-shaped iron core (Fig. 1A), which can generate a vertical sinusoidal MF with a magnetic flux density of up to 400 mT (rms) at 50 Hz. The MF was measured using a Gaussmeter (Model 9550; F.W. Bell, Orlando, FL, USA) and determined to show > 95% uniformity. A sample stand was installed in the middle of the coils. The stand was fixed on the foundation independent of the coils to exclude vibration artifacts. An acrylic chamber (Fig. 1B) was also placed on the stand. The chamber has separate thin spaces on the top, bottom, and rear for circulating heated fluorocarbon inactive solution (Fluorinert; 3M, St. Paul, MN, USA), and the internal temperature was maintained at $37 \pm 0.5^\circ\text{C}$. During the MF exposure tests, an iPS-CM monolayer on an MEA probe, which was placed in a connector with a cable, was positioned in the chamber. All of the metallic components of the connector, with the exception of the circuit board, cable plug, and cable connector, were replaced with acrylic substitutes. After one hour of acclimatization, the FPs of the iPS-CMs were recorded for

3 min. The cells were exposed sequentially to a 50 Hz MF at 0 and 400 mT for 9 sec (3 sec of 0 to 400 mT, 3 sec of 400 mT, and 3 sec of 400 to 0 mT), followed by 3 min of recording of the iPS-CM signals. The 9-sec MF exposure was controlled by a function generator (WF1974; NF, Yokohama, Japan). Finally, the iPS-CMs were incubated

Fig. 1. Magnetic field (MF) exposure system. A: A pair of saddle-shaped coils and a window frame-shaped iron core. B: Acrylic chamber for temperature control.

Fig. 2. Differentiation of human induced pluripotent stem (hiPS) cells into cardiomyocytes (CMs). **A:** Schematic of the differentiation protocol. After formation of embryoid bodies (EBs) from hiPS colonies, the EBs were attached to gelatin-coated culture dishes. During the cultivation period, beating CMs derived from hiPS cells (hiPS-CMs) were mechanically isolated under a microscope and used for further experiments. **B:** Immunofluorescence staining of hiPS-CMs. Fabricated hiPS-CM monolayers were stained with antibodies against the cardiac-specific proteins α actinin, GATA4, troponin T, ANP, MHC, and Nkx2.5.

with 200 nM of E-4031 for 15 min followed by recording the FPs for 3 min (Fig. 4A). The magnetic flux density in the exposure space was confirmed using a Narda ELT-400 with 3 cm² probe (Pfullingen, Germany).

Statistical analysis

Data are expressed as means $\pm$ standard deviation (S.D.), and significant differences between groups were determined using the 2-sample Student's *t*-test. P-values < 0.05 were considered statistically significant.

RESULTS

Differentiation of human iPS cells to cardiomyocytes

We induced differentiation of human iPS cells to cardiomyocytes using a general method for EB formation (Fig. 2A). Some of the EBs started beating spontaneously approximately 14 days after differentiation. Initially, to characterize the induced cells, we prepared a monolayer of beating EBs and performed immunofluorescence staining. The cells were positively stained with antibodies

directed against the cardiac-specific transcription factors GATA4 and Nkx2.5 and the cardiac structural proteins α actinin, myosin heavy chain (MHC), and troponin T (Fig. 2B). Sarcomeric organization with well-organized cross-striation and atrial natriuretic peptide (ANP) in the secretory granules surrounding the nuclei were also observed. These results confirmed the formation of a hiPS-CMs monolayer.

Pharmacological features of human iPS cell-derived cardiomyocytes

We next evaluated the pharmacological features of the hiPS-CMs. Changes in the FPs of hiPS-CMs following drug loading were monitored using an MEA system. FP duration (FPD), which is the time interval from the depolarization spike to the peak of the repolarization phase, was measured (Fig. 3A). The FPD is known to correspond to the duration of the action potential, which reflects the QT interval in an electrocardiogram (Reppel *et al.*, 2007; Halbach *et al.*, 2003; Caspi *et al.*, 2009). Figure 3B indicates that the I_{kr} channel blocker E-4031 prolonged the FPD in a concentration-dependent manner and the calcium channel blocker verapamil shortened the FPD concentration-dependently. The same changes of FPD were confirmed following each drug loading in two other independent experiments. Thus, the induced hiPS-CMs exhibited normal pharmacological sensitivity.

Responses of human iPS cell-derived cardiomyocytes to 50 Hz magnetic fields

To study the responsiveness of hiPS-CMs to power-frequency MF, we exposed a hiPS-CM monolayer on an MEA probe to a 50 Hz MF and simultaneously recorded the FPs of the cells pre- and post-exposure (Fig. 4A). Representative FPD data collected from a single sample exposed to a 50 Hz MF are shown in Fig. 4B. The FPDs of pre-exposed hiPS-CMs were not significantly altered by exposure to a 50 Hz MF at 400 mT for 3 sec (Fig. 4C). The MF-exposed hiPS-CMs were confirmed to exhibit normal sensitivity to E-4031 (Fig. 4C). The same results were observed in two other independent experiments with different hiPS-CM samples. These results suggested that short-term exposure to a 50 Hz MF at 400 mT does not disrupt the electrical activity of cardiomyocytes.

DISCUSSION

Here, we evaluated the stimulant effect of power-frequency MF on the electrical activity of human CMs using CMs differentiated from hiPS cells. HiPS-CMs have recently attracted considerable attention, not only

Fig. 3. Characterization of hiPS-CMs. A: Extracellular field potential (FP) of a hiPS-CMs monolayer recorded using a multi-electrode array (MEA) system. The time interval from the depolarization spike to the peak of the repolarization phase was used as the FP duration (FPD). B: Drug loading of the hiPS-CMs monolayer. After loading each concentration of a stock solution of each drug, the FP of the hiPS-CM monolayer was recorded and changes in the FPDs were analyzed. Each bar represents the mean and SD of all FPDs for each recording time. * P < 0.01.

Fig. 4. Responses of hiPS-CMs to a 50 Hz magnetic field. A: Experimental design for magnetic field (MF) exposure and field potential (FP) recordings. A hiPS-CMs monolayer was exposed to a 50 Hz MF at 400 mT and the FPs were recorded pre- and post-exposure. After MF exposure, the pharmacological sensitivity of the sample was confirmed. B: FP duration (FPD) recorded from a sample. Although electrical noise was generated during initiation of the MF exposure system, the FPs were recorded continuously through each experiment. C: Comparison of FPDs pre-exposure, post-exposure, and following addition of E-4031. Each bar represents the mean and SD of all FPDs in each period recorded from a sample.

for their potential medical uses for recovery of cardiac function, but also for their potential pharmaceutical uses. Cardiac activity is known to differ between humans and experimental animals. Beating rate and electrocardiogram results differ greatly between species, as do the molecular mechanisms of repolarization and expression of various genes and proteins (Boukens *et al.*, 2014; Denning *et al.*, 2016). These cause differences in each species' tolerance to chemical agents (Price *et al.*, 2008), and might yield false-positive or -negative results when human toxicity is evaluated using experimental animals and cells. Tests using many kinds of known cardioactive compounds have shown that hiPS-CMs are useful for evaluation of pharmacological responsiveness in humans (Sirenko *et al.*, 2013; Khan *et al.*, 2013). Thus, assay systems using hiPS-CMs are likely to provide accurate and efficient screening of candidate drugs. HiPS-CMs also show potential as a powerful tool to evaluate biological effects in health risk assessment. In this study, we applied hiPS-CMs to evaluate the effects of power-frequency MFs. To the best of our knowledge, this is the first report in which cells derived from iPS cells were used for assessment of human responses to non-ionizing radiation.

The effects of low-frequency EMFs on the electrical activity of CMs have previously been evaluated *in vivo* in dogs and rats and *in vitro* in mouse and rat CMs (Cornacchione *et al.*, 2016; Wei *et al.*, 2015). Despite the importance of gathering human-specific data, given interspecies differences in cardiac activity, few studies for risk assessment have been performed on human tissues. Some studies in which the strength of EMF exposure was sufficiently low to be safe, have been performed in humans (Cook *et al.*, 1992; Korpinen *et al.*, 1993; Graham *et al.*, 1994; Korpinen and Partanen, 1994). In this study, hiPS-CMs were subjected to short-term exposure to a 50 Hz MF at 400 mT, and the results suggested that under these conditions, MF exposure does not affect the electrical activity of hiPS-CMs. The flux intensity of MF applied to cardiomyocytes in this study is higher than that in previous reports. Simple calculations based on the positions of the electrodes on an MEA probe indicated that the maximum strength of the induced electric fields to which the hiPS-CMs were exposed in this experiment was approximately 380 mV/m, which is approximately 4-fold higher than the basis for restriction (90 mV/m at 50 Hz) specified in the ICNIRP guidelines (International Commission on Non-Ionizing Radiation Protection, 2010). In the IEEE standard, the median *in situ* induced electric field strength threshold for cardiac excitation is set at 12 V/m at 50 Hz (The Institute of Electrical and Electronics Engineers, 2002). The data reported here are valuable for experimen-

tally verifying the safety and reliability of these guidelines and standard values, which are based on theoretical models. It is expected that future experiments using hiPS-CMs and stronger MF exposure would give us more reliable threshold values of the effects of 50 Hz MF on human CMs.

The effect of low-frequency EMFs on intracellular calcium transients of CMs has been reported previously (Sert *et al.*, 2011; Fixler *et al.*, 2012). Low-frequency EMFs were found to change intracellular calcium concentration levels and the frequency of calcium transients in nonhuman CMs. Based on the strong correlation between the results of intracellular calcium imaging and those of extracellular FP recordings (Fujiwara *et al.*, 2008), MFs of 50 Hz at 400 mT might not affect intracellular calcium transients in hiPS-CMs. This discrepancy might be explained by interspecies differences described above. However, it should be mentioned that we evaluated the influence of MFs on hiPS-CMs by comparing the FPs of pre- and post-exposure cells. We were not able to analyze the FPs of cells during MF exposure because of the noise generated during MF exposure. Recently, we developed a non-conductive, miniature fiber-optic imaging system (Saito *et al.*, 2017). This system enables real-time imaging of intracellular calcium transients in electrically activated cells, such as neurons and CMs, during MF exposure without any artifacts. If real-time imaging of hiPS-CMs activities in MFs is performed with this system, previously undetectable phenomena might be able to be detected.

In conclusion, valuable knowledge about the effects of power-frequency MFs on the electrical activity of human CMs was acquired using hiPS cell technology. HiPS-CMs allowed evaluation of human responsiveness to strong MFs, which is impossible to perform using primary animal cultures, experimental animals and cells, or humans. Various types of human cells derived from iPS cells could be utilized for assessment of the health risks of various targets, including EMFs. The current international guidelines/standards for low-frequency EMF exposure deserve further investigation on many points, and experimental data on human neural responsiveness to EMFs have attracted much interest in recent years (Legros *et al.*, 2012, 2015). We propose that data on muscles, in particular CMs, should be similarly collected for evaluation of the risk of exposure to EMFs.

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

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