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Effect of 50-Hz Sinusoidal Magnetic Field on the Production of Superoxide Anion and the Expression of Heat-shock Protein 70 in RAW264 Cells

Marootpong Pooam[1,2], Maresuke Nakayama[2], Chihiro Nishigaki[2], Hidetake Miyata[2]

[1]Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, 65000, Thailand

[2]Department of Physics, Tohoku University, Aramaki, Aza-Aoba, 6-3, Sendai 980-8578, Miyagi, Japan

Correspondence: Hidetake Miyata, Department of Physics, Tohoku University, Aramaki, Aza-Aoba, 6-3, Sendai 980-8578, Miyagi, Japan. E-mail: miyata@bio.phys.tohoku.ac.jp

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Abstract

There is a growing concern if the power-line frequency (50/60 Hz) magnetic field (termed in this paper ELF-MF) increases cancer risks. Since one of the major causes of cancer is cellular oxidative stress, whether the ELF-MF increases the oxidative stress is a central problem in the studies on the biological effect of the ELF-MF. Here, we have investigated the effect of 50-Hz sinusoidal magnetic field on the production of O_2^-, the expression of heat shock protein (HSP) 70 and the mitochondrial membrane potential in cell line macrophage RAW264 cells. Macrophages were exposed to or not exposed to 0.1-mT or 0.5-mT, 50-Hz sinusoidal magnetic field and were subjected to (1) assay for O_2^- (2) analysis of the expression of HSP70, and (3) measurement of the mitochondrial membrane potential with a fluorescent indicator. The 50-Hz magnetic field enhanced production of O_2^- and the expression of HSP70, both of which are consistent with previous studies. The exposure to 50-Hz magnetic field decreased mitochondrial membrane potential indicating the diminished activity of mitochondria. The uncoupler of mitochondrial function, carbonyl cyanide p-trifluoromethoxyphenylhydrazone diminished the membrane potential, as expected. On the other hand, it increased the production of O_2^-. The results collectively suggest that the 50-Hz magnetic field diminished the mitochondrial membrane potential, which led to the increase in the production of O_2^- and the expression of HSP70 protein.

Keywords: extremely low frequency magnetic field, oxidative stress, mitochondria

1. Introduction

The rapid development of telecommunication technology in recent years is accompanied by an explosive increase in the number of electronic devices. This is only one consequence of the ever-increasing usage of electricity in every aspect of human activities. Along with this, the power lines are spreading all over the city areas. The increasing electrical activities contribute to the generation of electromagnetic fields with a wide range of frequencies and intensities. The biological effect of the environmental electromagnetic fields has been a public concern during a past few decades, because the increase in health risks such as childhood leukemia or brain tumor have been repeatedly pointed out (the International Commission on Non-Ionizing Radiation Protection (ICNIRP), 2010); Report of partial findings of National Toxicology Program, 2016). The World Health Organization (2007) has classified ELF-MFs as class 2B, a possible carcinogen to humans.

For over several decades, researchers have studied the in vitro effects of the ELF-MF on various types of the cell under a wide variety of conditions of the exposure to elucidate the mechanism of interaction between living-organisms and the ELF-MF (typically 50/60 Hz magnetic fields originating from power lines and house appliances; eg., Goodman and Blank, 2002; Simkó and Mattsson, 2004; Singh and Kapoor, 2014). Some studies have shown that the ELF-MF with appropriate intensity and frequency alters the free radical homoeostasis in the cell (Henrykowska et al., 2009; Mannerling et al., 2010; Lin and Lin, 2011; Poniedzialek et al., 2013). It has been suggested that a consequence of the altered balance is DNA strand breaks (Lai and Singh, 2004; Ivancsits et al., 2002; Nakayama et al., 2014, 2016). These studies have led to the suggestion that the ELF-MF affects the cellular oxidative stress, although the relation of this effect to clinical symptoms such as headache, skin symptoms, allergy-like symptoms, fatigue, etc. (Hojo et al., 2016) has remained unsubstantiated.

The oxidative stress is one of various types of stress such as high temperature, extreme pH, osmotic pressure, heavy metals (eg., Cd^{2+}) and alcohol, which are deleterious to the cell (Santoro, 2000). Heat shock proteins (HSPs) are

activated when the cell is exposed to the stress. Important roles of HSPs are to prevent misfolding of nascent proteins and to promote degradation of denatured proteins that are generated in the cell undergoing the stress, so that the damages to the cell is minimized or the recovery of the cell from damages is promoted (Jolly and Morimoto, 2000). It has been postulated that the 60-Hz ELF-MF alters the transcription and translation of HSP70 gene in HL60 cells (Lin et al., 1997, 1999, 2001; Blank and Goodman, 2009; Rodríguez de la Fuente et al., 2009) by activating the promotor region that is distinct from the region activated in response to the heat (Taira et al., 1992). Other studies have also reported the increase in the production of HSP70 by the ELF-MF in HL-60 cells (Pipkin et al., 1999; Tokalov and Gutzeit, 2004) or in THP-1 cells (Akan et al., 2010). On the other hand, there are studies showing no increase in HSP70 protein (Morehouse and Owen, 2000; Gottwald et al., 2007), but Gottwald et al's study has demonstrated an increase in the mRNA level in HL-60 cell. Another study (Alfieri et al., 2006) has suggested that the increase in the HSP70 protein by the ELF-MF depended on the cell type and the intensity of the magnetic field. Thus, the effect of the ELF-MF on the expression of HSP seems to depend on differences in the experimental conditions or cell strains. In vivo studies have demonstrated the expression of HSP70 by the ELF-MF in *Mytilus galloprovincialis* (Malagoli et al., 2004) or in Planaria *Dugesia dorotocethala* (Goodman et al., 2009).

That an increase in the level of expression of HSP is a consequence of the elevated cellular oxidative stress (McDuffee et al, 1997; Morimoto et al., 1998) is consistent with the numerous results demonstrating that the ELF-MF exposure enhances the oxidative stress. Thus, the ELF-MF was likely to promote the HSP expression by increasing the oxidative stress. Establishing the connection between the ELF-MF, oxidative stress and HSP70 will lead us to deeper understanding of the effect of ELF-MF on the physiology of the cell. On the other hand, it has been shown that ELF-MF changes the membrane potential of mitochondria in C2C12 or GL15 cells exposed to 1 mT, 50-Hz magnetic field for 30 min (Farina et al., 2010), but the relation of this phenomenon to other effects of the ELF-MF has not been well understood. Here, we show the effect of ELF-MF on the intracellular concentration of superoxide anion (O_2^-), the expression of HSP70 and the mitochondrial membrane potential in the cell line macrophage RAW264.7. Since macrophage is abundant in the human body and plays important roles in immunological responses, the in vivo consequence may be significant, if the free radical homoeostasis and the expression of HSP70 in macrophages are changed by the ELF-MF.

2. Materials and Methods

2.1 Cell

Leukemic monocyte cell-line cell (macrophage RAW 264.7) was obtained from Riken, BioResource Center (RBRC-RCB0535; Wako, Saitama, Japan) and was used throughout.

2.2 Chemicals

Minimum Essential Medium (MEM), fetal bovine serum (FBS), trypsin, penicillin-streptomycin, L-glutamine were purchased from Gibco© Life Technologies (Tokyo, Japan). Acrylamide, (*N*,*N*'-methylenebis)acrylamide, sodium dodecyl sulfate (SDS), glycine, ammonium peroxodisulfate, tetramethylethylenediamine, β-mercaptoethanol, bromophenol blue (all used for sodium dodecyl sulfate polyacrylamide gel electrophoresis), amido-black 10B, skim milk, polyoxyethylene (9) octylphenyl ether (NP40), potassium hydroxide, dimethyl sulfoxide, methanol and ethanol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Osaka, Japan). A 2-Amino-2-[hydroxymethyl]-1,3-propanediol (tris) was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Anti-actin primary antibody and horse radish peroxidase-conjugated anti-immunoglobulin G (IgG) secondary antibody were purchased from Santa Cruz Biotechnology (I-19 and sc-3837, respectively; Dallas, TX, USA). The rabbit primary antibody against HSP70 was obtained from Cell Signaling Technology (D69; Danvers, MA, USA). Nitro blue tetrazolium chloride (NBT) was purchased from Dojindo Laboratories (Kumamoto, Kumamoto, Japan). Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was obtained from Abcam (Tokyo, Japan). Pierce™ BCA protein assay kit and Halt™ protease inhibitor cocktail were obtained from Thermo Scientific (Rockford, IL, USA). Marker Gene™ Dyrect JC-1 Mito Health Assay Kit was obtained from Marker Gene Technologies, Inc. (Eugene OR, USA). Amersham™ ECLstart western blotting detection reagent was purchased from GE Healthcare UK Ltd. (Buckinghamshire, England). Polyoxyethylene sorbitan monolaurate (Tween 20) was from Polysciences Inc. (Warrington, PA, USA). Nitrocellulose membranes (pore size 0.2 μm) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma Aldrich Japan (Tokyo, Japan).

2.3 Cell Culture

Cells were cultured in a CO_2 incubator (NAPCO model 5410, Precision Scientific, Chicago, IL, USA) at 37 °C, 5% CO_2 and at 90% humidity; the culture medium was MEM supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin-streptomycin. Cells were sub-cultured every 2 days in 6-cm or 10-cm petri dish (AGC Techno Glass, Shizuoka, Japan). Cell number was counted by Neubauer haemocytometer (Hirschmann, Eberstadt Germany).

2.4 Magnetic Field Exposure

A 1×10^7 cells were cultured in 10-cm petri dishes (for HSP70 assay and O_2^- assay) for 3 days or 6×10^5 cells in 6-cm petri dishes (for the assay for mitochondrial membrane potential) until the day of the start of the exposure to the 50-Hz sinusoidal magnetic fields (hereafter, 50-Hz MF).

All the exposure/ sham-exposure were carried out in two CO_2 incubators of the same make (Model IT600, Yamato Scientific Co., Tokyo, Japan), in which the CO_2 concentration was maintained at 5%, the humidity at 90% and the temperature at 37 °C. For the exposure, two Merritt coil systems (Merritt et al., 1983) were constructed (Hozen Industries Co., Ltd., Kyoto, Kyoto, Japan) and used. Each Merritt coil system was comprised of four sub-coils, each of which was constructed with a pair of electrical wires wound around an aluminum frame. The shape of the coil frame is given in Figure 1(A) and its dimension is described in the legend to Figure 1(A). The top and the bottom coils were made from 26 turns of the parallel electrical wires and the middle two coils were made of 11 turns of the parallel electrical wires, respectively. Each coil system was placed in the CO_2 incubator. One coil system in one incubator was used for the exposure, in which the electric current in the two wires ran in parallel (the sample in this coil is hereafter called the exposed sample). The other coil system in another incubator was simultaneously used for the sham-exposure, in which the electric current ran in antiparallel (the sample in this coil is called the sham-exposed sample). The 50-Hz sinusoidal electric current was generated by function generators (Model WF1973 or WF1943A, NF Corporation., Yokohama, Kanagawa, Japan), amplified with electric amplifiers (PMA390AE or PMA390SE, DENON, Kawasaki, Kanagawa, Japan), and was fed to the Merritt coil system. Owing to the parallel, double-wiring configuration, the Merritt coil system used for the exposure and the sham-exposure both generated the same amount of Joule heat and the difference in the temperature between two groups was at most 0.2 °C, according to the temperature record made with Thermo Recorder equipped with a thermistor probe (TR-71Ui, T&D, Matsumoto, Nagano, Japan; Nakayama et al., 2014, 2016).

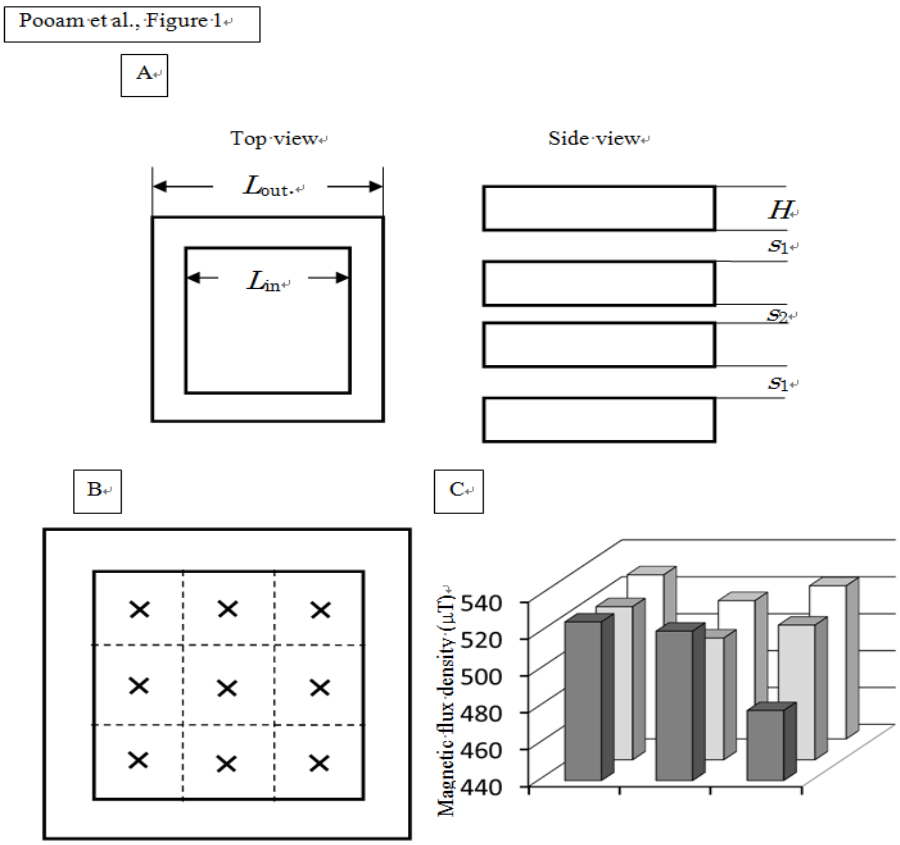

Figure 1. (A) Design of a Merritt coil used in this study. The Merritt coil was consisted of four identical square aluminum frames of which outer edge (L_{out}) was 20 cm, inner edge (L_{in}) was 16 cm and height (H) was 4 cm. Frames were supported by a plexiglass frame (not drawn); the spacing (s_1) between the top and the second frames and that between the third and the bottom frames was 4 cm; the spacing (s_2) between the second and the third frames was 2 cm. The top and the bottom frames were wound with 26 turns and the two middle frames were wound with 11 turns of a parallel electrical wire. (B) The nine locations in the Merritt coil (top view), where 50-Hz MFs were measured; the vertical position was approximately the bottom of the second frame. (C) A 3-D plot of the values of the magnetic flux densities measured at each point as shown in the panel (B). Note the scale of the vertical axis.

At the 0.5-mT setting, the flux density in the exposure coil was 0.515 ± 0.016 mT (average ± standard deviation measured at 9 points as shown in Figure 1(C)). Considering the dimensions of the 6-cm cell culture dishes or 1.5-mL microtubes that were placed around the center of the coil, we presume that the cells in the culture dish or in the microtubes were exposed to fairly uniform magnetic fields.

The background AC magnetic field in the sham-exposure and the exposure coil, both measured in the active-sham mode at the center of the coil with a Gauss meter with a frequency range of 20 Hz to 2000 Hz (FW4190, Pacific Scientific-OECO, Milwaukee, OR, USA) were 0.2 μT and 0.3 μT. When coils were turned off, the background AC magnetic fields in the sham-exposure and the exposure coil, measured at the center of the coil with the same Gauss meter were both 0.2 μT. The background DC magnetic fields of the sham-exposure coil and that for the exposure coil were 3 μT and 35 μT as measured at the center of the coil with a Gauss meter (Model 421; Lake Shore, Westville, OH, USA) equipped with a Hall-effect probe (MMA-2502-VH).

Prior to the measument of O_2^- or mitochondrial membrane potential, cells cultured in petri dishes were dissociated with a rubber policeman and were distributed in six1.5-mL microtubes (three for the active-sham and three for the exposed sample). Microtubes were then placed in the Merritt coil system. For the measurement of the expression of HSP70 protein, 10-cm petri dishes, in which cells had been cultured, as described above, were placed in each Merritt coil (one in each coil). Center of the dishes was adjusted to coincide with the center of the coil; the microtubes were placed around the center of the coil. In all the experiment the locations near the coil frame were avoided. The vertical position was also adjusted with a rack made of plexiglass so that the plane of the cell matched the center of the coil system.

We adopted the exposure conditions for following reasons. The exposure of human leukemica cell (K562) to 50-Hz, 0.025-mT - 0.1-mT MFs for 1 h resulted in the increase in O_2^- production and the expession of HSP70 (Mannerling et al., 2010). The exposure of fibroblasts or RAW264 cells to 0.5-mT 50-Hz MF for 24 h has been shown to increase necrosis and DNA single-strand breaks (Nakayama et al., 2014, 2016). The duration of 17 h was adopted, because the doubling time of the macropahge was measured to be 16.8 ± 0.11 h (a duplicated measurement).

2.5 Analysis of the Intracellular Concentration of O_2^-

The intracellular concentration of O_2^- was measured with NBT according to the previously described method (Choi et al., 2006). Cells were sub-cultured in 1.5 ml centrifuge tubes overnight and were subjected to exposure/sham-exposure. Immediately after the exposure, cells were collected by centrifugation (28 × g for 5 min) and the pelleted cells were incubated in MEM supplemented with 1 mM $CaCl_2$ and 1 mM NBT for 1 h. After this step, cells were collected by centrifugation (28 × g for 5 min) and were washed twice with phosphate-buffered saline (PBS: 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}12H_2O$, 1.47 mM KH_2PO_4, pH 7.2). Then, cells were collected with centrifugation (10,000 × g for 5 min) and supernatant was removed and the pelleted cells were air-dried. Blue formazan crystals in the pellet were dissolved, first in 50 μl of 2 M KOH for 10 min followed by addition of 100 μl dimethylsulfoxide for 10 min with shaking. Finally, the NBT solution was transferred to 96-well microplate (AGC Techno Glass, Shizuoka, Japan) and the absorbance at 620 nm (A620, hereafter) was measured with a microplate reader (Model SH-9000, Corona Electric, Hitachinaka, Ibaraki, Japan).

Because A620 values of the exposed, sham-exposed, heat-treated and PMA-treated samples considerably varied among the measurements, the A620 of un-treated cell that was left in the normal CO_2 incubator, not subjected to the above treatments (named hereafter, Control), was measured and is presented as a reference along with the result of the treated samples. We also subjected cells to heat treatment (42 ºC for 30 min) or the stimulation with PMA (600 ng/ml for 30 min; Lupke et al., 2004) as positive controls for O_2^- production.

Measurement under each condition was triplicated and each measurement was repeated three times. As shown with striped bars in Figure 3(B), the A620 value of the Control varied among the experiments performed on different days. We suspect that this was due to the difference in the passage of the cell. This precluded us from comparison of the result obtained in the experiments performed on different days. Thus, the A620 values of the exposed samples and the sham-exposed samples obtained on the same day were compared to each other using paired t-test (IBM SPSS Statistics 23; IBM Corporation.,New York, USA).

2.6 Western Blotting Analysis of the Expression of HSP70

Immediately after the exposure, cells were collected by centrifugation (28 × g, 5 min) and were washed with PBS and finally pelleted by centrifugation (10,000 × g for 5 min) at room temperature. Cells were then incubated at 4 ºC for 30 min in lysis buffer (50 mM tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, supplemented with the Halt™ protease inhibitor cocktail). The cell lysate was measured for protein concentration with BCA protein assay, mixed with the sample buffer (62.5 mM, tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue and 2% β-mercaptoethanol) at 1:1 dilution and heated at 95 ºC for 5 min.

Gel electrophoresis was carried out in a separation gel (12% polyacrylamide + 0.3% bisacrylamide) and the stacking gel (4% polyacrylamide + 0.1% bisacrylamide) with the buffer system (0.25 M tris, 0.192 M glycine and 0.1% SDS) based on the method developed by Laemmli (1970) on a mini-gel apparatus (NA1013, Nihon Eido, Tokyo, Japan). About 40 micrograms of total protein was loaded to each well formed in the stacking gel. The electrophoresis was carried out at 120 V, 35 mA (Crosspower1000, Atto Corporation, Tokyo, Japan) for 90 min. Proteins thus separated on polyacrylamide gel were transferred to a nitrocellulose membrane (pore size = 0.2 μm; Bio-Rad, Hercules, CA, USA) by using a blotting apparatus (Trans-Blot®; Bio-Rad). The electrophoretic transfer was carried out at 25 V, 200 mA for 60 min with a transfer buffer (25 mM tris, 250 mM glycine and 0.1% SDS). The efficiency of protein transfer was checked by amido-black staining of the separate membrane. After that, the membrane was incubated with the blocking solution (5% skim milk plus 0.1% Tween-20 in PBS) at 4 °C overnight. Membrane was then incubated for 90 min with the primary antibody for HSP70 at a 1:500 dilution and that for actin at a 1:300 dilution in PBS supplemented with 0.1% Tween-20. Then, the membrane was washed three times, each for 15 min, in washing solution (0.5% Tween-20 in PBS) and was incubated for 90 min with the horse radish peroxidase-conjugated secondary antibody at a dilution of 1:2000. After the reaction, the membrane was washed four times, each for 30 min, with washing solution. Protein bands were visualized by chemiluminescence technique according to the manufacturer's instruction. For the acquisition of the image of the luminescent bands, a cooled CCD camera (C4742-89-12AG, Hamamatsu, Shizuoka, Japan) was attached to the dark box eqipped with a UV illuminator (BioDoc-IT®; UVP, Analytik Jena AG, Jena, Germany). The band images were acquired with 4-min exposure, and the band intensity was quantified with ImageJ 1.50i software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA). The ratio of the band intensity of HSP70 in each lane was divided by the intensity of actin band in the same lane (expression ratio; Akan et al., 2010).

In each experiment, measurements were triplicated and the experiments were repeated three times. For the same reason as described in the preceding section, we did not perform the comparison of the data obtained on different days. The averaged expression ratios of the exposed and the sham-exposed samples were compared with paired t-test.

2.7 Assay for Mitochondrial Membrane Potential

The membrane potential of mitochondria was assayed with the fluorescence probe, JC-1 (Reers et al., 1991). In a mitochondrion, this probe localizes to the mitochondrial matrix according to the magnitude of the membrane potential. The peaks of the emission spectrum change depending on the degree of the aggregation of the probe. Thus, higher degree of aggregation arising from the higher concentration of JC-1 is reflected by the emission peak with longer wavelengths; the higher concentration of JC-1 in mitochondrial matrix is achieved in mitochondria with higher membrane potential (higher activity). Immediately after the exposure, the probe was added to the cell suspension according to the manufacturer's instruction. Then, the emission peak intensity at 600 nm and that at 540 nm, both obtained with the excitation at 488 nm, were measured in the microplate reader. To quantify the ratio of the aggregate to the monomer, and the emission ratio (the emission intensity at 600 nm/ the emission intensity at 540 nm) was calculated (Perelman et al., 2012). Higher emission ratio represents higher degree of aggregation of the probe, and hence, higher activity of mitochondria. To confirm that the loss of membrane potential results in the decrease in the emission ratio, we utilized FCCP that decouples the respiratory chain from ATP synthesis in mitochondria (Benz and McLaughlin, 1983). FCCP was added to the cell suspension, which was followed by incubation at 37 °C for 30 min and the fluorescence ratio was measured with the microplate reader.

Experiment under each condition was duplicated and the experiments were repeated 2 to 4 times. As described in section 2.5, the average of the emission ratios of the exposed and the sham-exposed samples, both of which were derived under each experimental condition, were compared to each other with paired t-test.

3. Results

3.1 Effect of the 50 Hz Magnetic Field on the Production of O_2^-

As shown in Figure 2(A), the A620 of the sample exposed to 0.1-mT 50-Hz MF for 24 h (white bar) was significantly higher ($p < 0.05$) than that of the sham-exposed sample (gray bar). In the case of 1-h or 17-h exposure the A620 of the exposed sample was not significantly different from that of the sham-exposed sample ($p > 0.05$). Figure 2(B) shows that the A620 value of the sample exposed to 0.5-mT 50-Hz MF for 1 h or 17 h was not significantly different from that of the sham-exposed sample, but for the 24-h exposure, the A620 value was significantly larger than the sham-exposed sample, when the sample was exposed for 24 h. Figure 2(C) shows that the A620 value of the cell treated with heat (42 °C for 30 min) was not different from that of the untreated cell (CON; $p > 0.05$); the A620 of the cell treated with 600 ng/mL PMA for 30 min was marginally larger than the untreated cell ($0.05 < p < 0.1$).

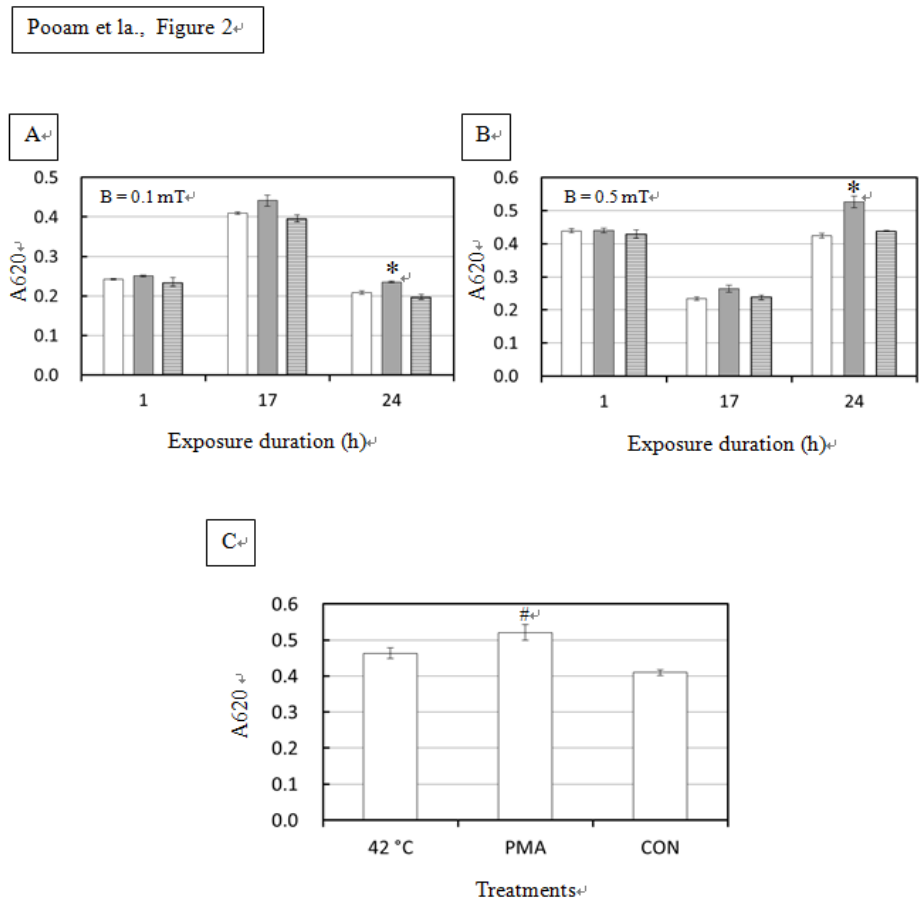

Figure 2. (A) The absorbance of NBT measured at 620 nm (A620) in RAW264 cells that had been exposed to 0.1-mT 50-Hz MF for 1 h, 17 h or 24 h. (B) The A620 for cells exposed to 0.5-mT 50-Hz MF for 1h, 17 h or 24 h. The A620 represents the concentration of O_2^-. Open bars represent sham-exposed samples and gray bars represent the exposed samples. Striped bars represent the values obtained with the sample left at 37 ºC in the CO_2 incubator during the exposure/ sham-exposure (Control). (C) Results of the exposure of the cell to high temperature (42 ºC) for 30 min or to a 600 ng/mL PMA (a phorbolester) for 30 min. The value obtained with the sample left at 37 ºC in the CO_2 incubator during the heat or PMA treatments (Control; CON) is also shown. The error bars represent the standard error of mean obtained from three independent experiments ($n = 3$), each carried out in triplicate. *$p < 0.05$.

3.2 Effect of the 50 Hz Magnetic Field on the Expression of HSP70

We next compared the level of the expression of HSP70 protein as estimated from the chemiluminescence intesity. Representative chemiluminescence images are shown in Figure 3(A). In each panel the upper bands represent HSP and the lower bands represent actin. Figure 3(B) shows the intensity ratios (ie., chemiluminescence intensity of HSP70 band/ chemiluminescence intensity of actin band), which were obtained with samples exposed to or sham-exposed to 0.1-mT 50-Hz MF for 1 h, 17 h or 24 h. The significant difference ($p < 0.05$) occurred only at 17 h exposure. In the case of the exposure to 0.5-mT 50-Hz MF, the sample exposed for 1 h or 17 h did not exhibit significant difference in the intensity ratio ($p > 0.05$). However, the intensity ratio of the sample exposed to 0.5-mT 50-Hz MF for 24 h was significantly higher than that of the sham-exposed sample ($p < 0.05$, Figure 3(C)).

We also examined the expression level of HSP70 in the cell incubated at 42 °C for 30 min as a positive control (Singh and Lakhotia, 2000). Unexpectedly, as shown in Figure 3(D), the level of expression of HSP70 was higher (1.11 times) than the Control group, which had been kept at 37 °C, but this difference was not statistically significant. This may be because we did not activate the macrophage with lipopolysaccharide. It has been shown that macrophages activated with lipopolysaccharide produce tumor necrosis factor-α and the level of HSP70 expression level increases 30 min after the heat treatment at 43 °C (Heimbach et al., 2001).

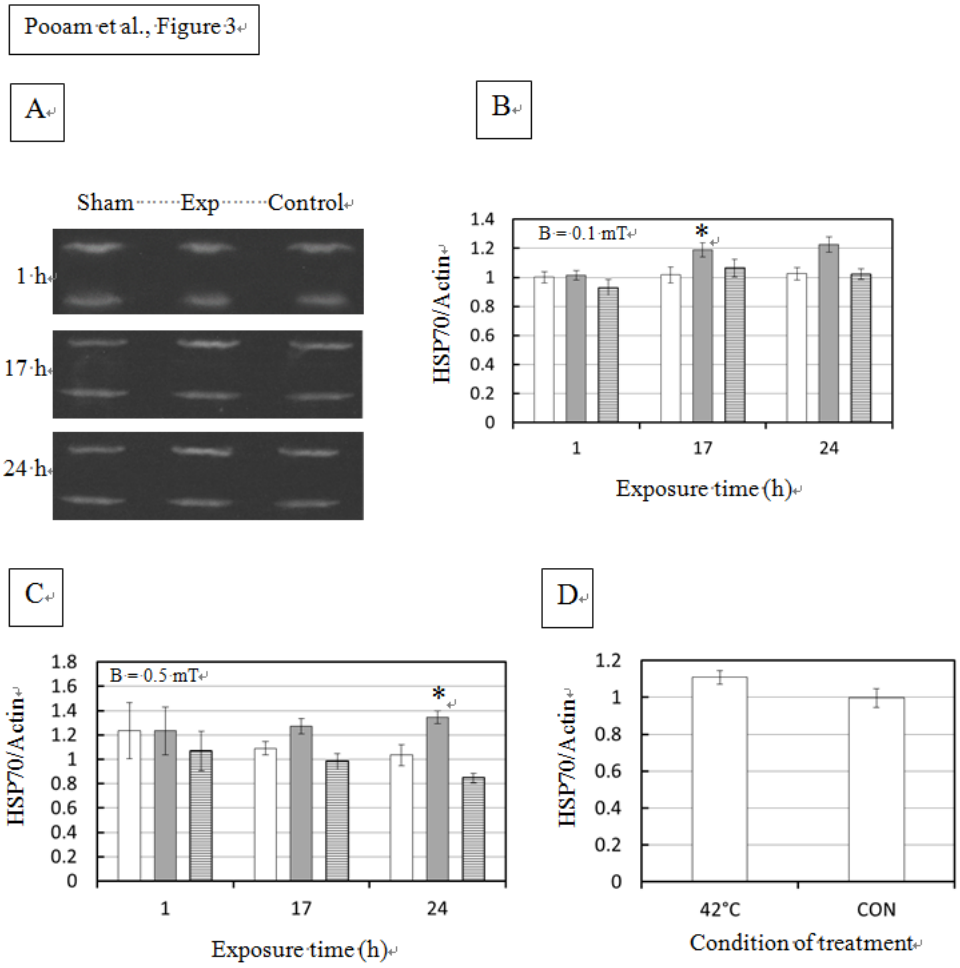

Figure 3. (A) Representative chemiluminescent images of the HSP70 and actin detected by Western blotting technique in the extract of RAW264 macrophage. Top panel, the result of 1-h, middle panel, 17-h and bottom panel, 24-h exposure. Control represents the cell left in the CO_2 incubator during the exposure/sham-exposure. In each panel, the upper bands represent HSP70 and the lower bands, actin. (B) A graph showing the ratio of the chemiluminescence intensity of the HSP70 band to that of actin band in the extract from the RAW264 cells exposed to 0.1-mT 50-Hz MF for 1 h, 17 h and 24 h and to (C) 0.5-mT MF for 1 h, 17 h and 24 h. In these panels, the active-sham results are represented with open bars and the exposure results are represented with gray bars. Striped bars indicate the results of Control. (D) The results of the heat treatment of the cell at 42 ºC for 30 min. Error bars represent the standard error of mean derived from the result of three independent experiments ($n = 3$), each measured in triplicate. *: $p < 0.05$.

3.3 Effect of 50-Hz MF on the Mitochondrial Membrane Potential

We examined the membrane potential of mitochondria in RAW264 cell, because one of the major sites of the production of O_2^- is the respiratory chain in mitochondria (Murphy, 2009). In the cell exposed to 0.1-mT 50-Hz MF for 17 h (Figure 4(A)), the emission ratio of JC-1 of the sample did not exhibit significant difference from that of the sham-exposed sample ($p > 0.05$, $n = 3$); likewise, 24 h exposure did not exhibit significant difference ($p > 0.05$, $n = 2$). In the cell exposed to 0.5-mT 50-Hz MF for 17 h (Figure 4(B)), the emission ratio did not exhibit significant difference from that of the sham-exposed sample ($p > 0.05$, $n = 3$); in the cell exposed for 24-h exposure the emission ratio exhibited a significant decrease ($p < 0.05$, $n = 4$).

An ionophore, FCCP, has been shown to decrease the membrane potential (Benz and McLaughlin, 1983). This was confirmed by the decrease in the emission ratio of JC-1 with FCCP concentration (Figure 4(C)). In a separate experiment, the production of O_2^- was found to become higher in the cell treated with increasing concentration of FCCP (Figure 4(D)).

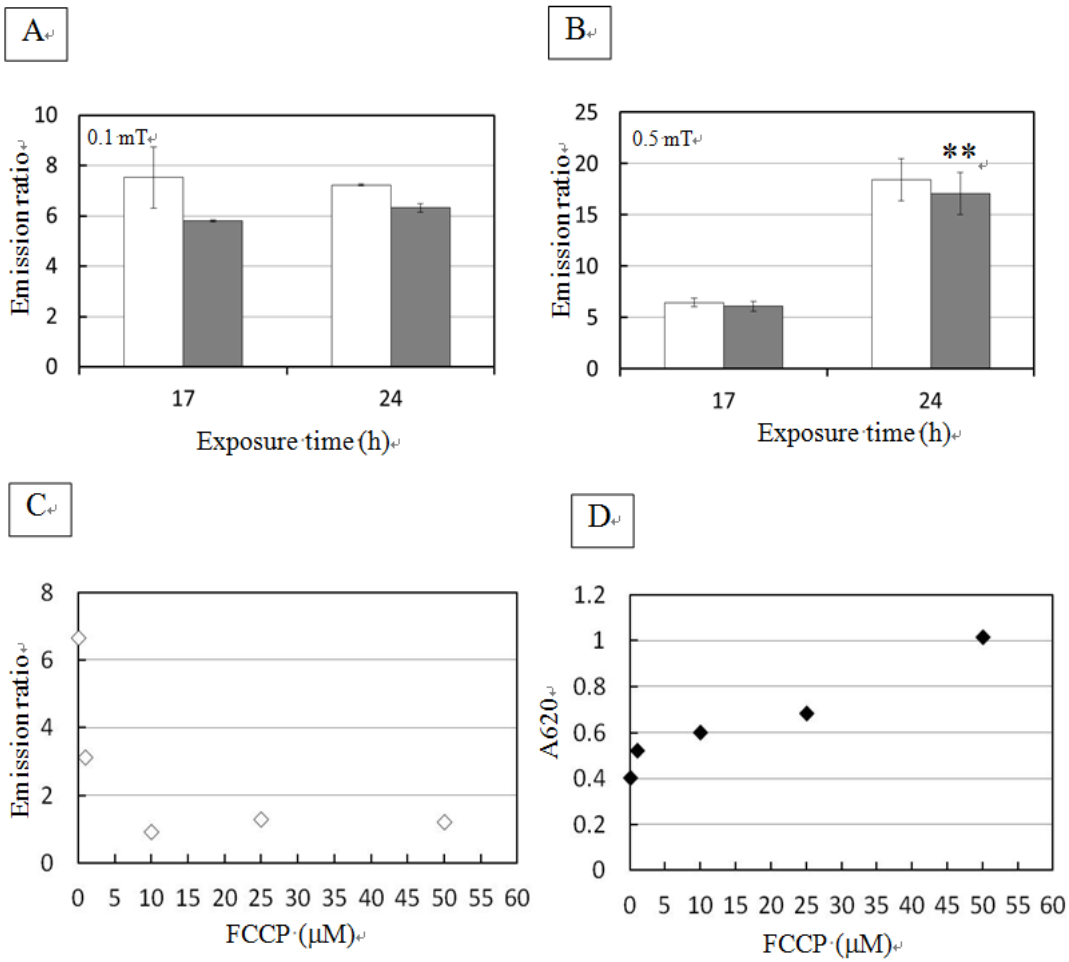

Figure 4. (A) Membrane potential of mitochondria represented as the emission ratio (the ratio of the emission intensity at 660 nm to that at 540 nm of JC-1). Cells were exposed (gray bars) to or sham-exposed (open bars) to 0.1-mT, 50-Hz for 17 h or 24 h. (B) The emission ratio measured after exposure to 0.5-mT, 50-Hz MF for 17 h or 24 h. Error bars represent the standard error of mean. **: $p < 0.01$. (C) The change in the emission ratio of the non-exposed cells treated with the increasing concentration of FCCP. (D) The change in the O_2^- (as represented with A620) in the non-exposed cells treated with the increasing concentration of FCCP.

4. Discussion

There are a number of redox reactions in the cell (Monti et al., 2011), in which radicals or radical pairs appear as intermediates. Radicals activate various signaling pathways that regulate a number of cellular processes such as gene expression and apoptosis (Buetler et al., 2004; Shen and Liu, 2005). Mitochondria produce reactive oxygen species including radicals during the process of oxidative phosphorylation. In this process, oxygen is reduced to H_2O via intermediate reactive oxygen species (H_2O_2, O_2^- and hydroxyl radical). Leakage of these reactive oxygen species can cause damage not only to mitochondrion itself (Murphy, 2009) but also to the cell by damaging cell membranes by lipid peroxidation, proteins by modifying amino acids and DNA by strand breaks (World Health Organization, 2007). Hence, anti-oxidant defense molecules such as superoxide dismutase, catalase, glutathione and melatonin must play pivotal roles in the maintenance of homeostasis of the cellular oxidative stress (Pham-Huy et al., 2008).

It has been postulated that the ELF-MF affects the cell functions or molecules related to the oxidative stress. This has been supported by a number of studies, eg., an increase in the O_2^- in cell line immune cells or in primary immune cells (Simkó et al., 2001; Rollwitz et al., 2004; Lupke et al., 2004; Frahm et al., 2006, 2010; Mannerling et al., 2010; Poniedzialek et al., 2013). Other studies have shown that the ELF-MF enhanced the exogenous oxidative stress by, for example, inactivation of hexokinase and depletion of reduced glutathione in red blood cells (Fiorani et al., 1997), or increased production of reactive oxigen species in blood platelets (Henrykowska et al., 2009). In one study brain cells were isolated from rats that had been exposed to 0.01-mT, 60-Hz MF for 24 h or 48 h (Lai and Singh, 2004). The result was an increase of DNA strand breaks; this effect was abolished in rats pre-treated with either vitamin E analog (Trolox), iron chelator (deferipron) or an inhibitor of nitric oxide synthase (7-nitroindazole). Based on these observations Lai and

Singh have proposed a model in which the ELF-MF caused a series of oxidative responses starting from the alteration of iron metabolism followed by an increase of nitric oxide and a subsequent elevation of the oxidative stress, a cause for the DNA strand breaks.

We have shown an increase in O_2^- production in RAW264 cells exposed to 50-Hz MF for 24 h at 0.1 mT or 0.5 mT. A number of studies have been conducted to elucidate the mechanism by which the ELF-MF affects the production of O_2^- in the cell. For example, it has been demonstrated that an exposure of Hela cells to electromagnetic field (875 MHz) only for a few minutes increased reactive oxygen species (Friedman et al., 2007). This has been attributed to an increase in the NADH oxidase activity. In another study an increase in the O_2^- production in murine bone marrow-derived phagocyte exposed to 50-Hz, 1-mT or 1.5-mT MFs for 45 min was independent of the phagocytic activity in which a large amount of O_2^- is produced (Simkó et al., 2001). Hence, in this case, the effect of the ELF-MF has been attributed to a direct interaction of the ELF-MF with the system of O_2^- metabolism. Another study has suggested that the increase in the free radicals in neutrophils exposed to 60-Hz, 0.1-mT MF was due to the increase in the lifetime of radical, not the production (Roy et al., 1995). Other studies have shown that the ELF-MF modulated the expression of superoxide dismutase (Mahmoudinasab et al., 2016), elevated the enzymatic activity of superoxide dismutase (Büyükuslu et al., 2006; Lee et al., 2004) or decreased the level of glutathione (Luukkonen et al., 2014). Thus, there seems to be a number of pathways (the production or the decompositon of O_2^- molecule) by which the ELF-MF can alter the concentration of O_2^-. There are several reports showing that the ELF-MF did not affect the production of reactive oxigen species (Hong et al., 2012; de Kleijn et al., 2011; Markkanen et al., 2010). The existence of the positive and the null effects by the electromagnetic fields is likely to stem from the complexity of the process of the O_2^- metabolism in the cell, as well as the differences in the cell type or the condition for the exposure such as field strength.

Our study has also shown that when RAW264 cells were exposed to 0.1-mT 50-Hz MF for 17 h or to 0.5-mT 50-Hz MF for 24 h, the expression level of HSP70 significantly increased. In a previous study (Malagoli et al., 2004) mussel immunocytes exposed to 50-Hz MF > 0.4 mT for various durations exhibited significantly higher expression of HSP70 than the control, but that did not occur < 0.3 mT. However, the level of HSP70 protein did not significantly increase in porcine aortic endothelial cells exposed to 1-mT 50-Hz MF (Bernardini et al., 2007), nor in HL-60 and two other cell types exposed to up to 3-mT 50-Hz MF (Gottwald et al., 2007). However, in the latter study, the elevation of HSP70 mRNA levels was found. There was no increase in the expression of HSP70 gene in Friend erythroleukemia cells exposed to 0.1-mT to 1-mT 60-Hz MFs (Chen et al., 2000), or in human leukocytes exposed to up to 0.1-mT 50-Hz MFs (Coulton et al., 2004). Thus, in a number of cases including ours, exposure to the ELF-MF seems to alter the HSP70 at the protein or genetic level, but one has to be cautious about generalizing the effect of the ELF-MF, because it clearly depends on the experimental conditions/ cell types.

Mannerling et al. (2010) has attributed the elevated expression of HSP70 protein in the cell exposed to the ELF-MF to the increased production of O_2^- by the ELF-MF. Our result that the exposure of RAW264 cells for 24 h to 0.5-mT 50-Hz MF resulted in the increase in the expression of HSP70 can be interpreted along the same line. Thus, in RAW264 macrophage cell exposed to 0.5-mT 50-Hz MF for 24 h, the expression of HSP70 protein increased probably as a result of the increase in the O_2^- production. However, at 0.1-mT flux density, the expression of HSP was found to increase in the cell exposed for 17 h, whereas the O_2^- increased after the exposure to 0.1-mT 50-Hz MF for 24 h. This discrepancy might be a result of some complex interaction of the ELF-MF with the up- or down-regulation mechanisms of O_2^- / HSP (Pirkkala et al., 2001). However, it is also possible that it was due to the relatively weak effect of the 50-Hz magnetic field.

Our results showed that the membrane potential of mitochondria decreased slighlty but significantly in RAW264 cells exposed to 0.5-mT 50-Hz MF for 24 h. We also showed that the membrane potential decreased in the presence of FCCP and that the same range of concentration of FCCP caused the increase in the O_2^- concentration. FCCP decreases the membrane potential and decouples the electron transport chain from the ATP syntehsis (Benz and McLaughlin, 1983; Brand and Nicholls, 2011). This would have caused the increase in the reducing activity in the electron transport chain, which then elevated the formation of O_2^-, as suggested from the experiments utilizing inhibitors for the components of the electron transport chain (Turrens, 2003). Our result obtained with FCCP is consistent with this notion. The membrane potential was not affected by the exposure to 0.1-mT, 50-Hz MF for 24 h. This might also be due to the weak effect of the 50-Hz MF. A recent meta-analysis of the effect of the ELF-MF on the oxidative response of the cell has suggested that the biological effect of magnetic field was consistently observed when the flux density was ≥ 1 mT (Mattsson and Simkó, 2014). In light of this study and our result, we conjecture that in the cells exposed to 0.5-mT 50-Hz MF for 24 h, a connection existed between the decrease in the membrane potential and the increase in the O_2^- formation.

The ELF-MF-induced expression of HSP70 may be applicable to the cytoprotection from the damage caused by ischemia. For example, HSP70 plays a role in reducing the damage caused by myocardial ischemia-reperfusion by

reducing the size of infarct and promoting the contraction (George et al., 2008). During ischemic injury caused by a decrease in the intracellular pH, ATP concentration or calcium overload, damages occur to the functions of proteins (Plumier and Currie, 1996). By interacting with cytoskeletal components, HSP70 promotes cytoskeletal-based cell survival and enhances the anti-apoptotic activity to improve the tolerance of the cell to ischemic injury (Wei et al., 2006). Until 24 h after the injury HSP70 alone does not provide the cell sufficient protection from permanent myocardial damage that arises from ischemia, but the ELF-MF has been shown to increase the expression of HSP70 after exposure at 8 mT only for 30 min (George et al., 2008). Likewise, the expression of HSP70 was found to increase after the exposure of rat heart to broad band electromagnetic fields, which seemed to lower the damage accompanying the ischemia-reperfusion (Ronchi et al., 2004). Thus, the clinical use of the electromagnetic fields against the ischemic injury will offer an advantage to both physician and patient, because this technique is non-invasive, imposing a very low level of energy. However, it is obvious that the effect should be further studied to determine the appropriate intensity and the duration of exposure for the proper clinical application.

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