Letter to the Editor

Protective Effect of 10-Hz, 1-mT Electromagnetic Field Exposure Against Hypoxia/Reoxygenation Injury in HK-2 Cells*

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We investigated the protective effects of electromagnetic field (EMF) on the survival of the human renal proximal tubular cell line, HK-2, using an *in vitro* hypoxia/reoxygenation (H/R) injury model. The survival rate of cells cultured under H/R condition declined significantly, while the intracellular reactive oxygen species (ROS) levels markedly increased. The 10 Hz/1 mT EMF exposure reversed the H/R induced reduction in cell survival and induction of intracellular ROS. Our results suggest that 10 Hz/1 mT EMF exposure could inhibit H/R-induced cell death of HK-2 *via* suppression of intracellular ROS production and that this treatment might be clinically useful for the amelioration of renal ischemia/reperfusion injury.

Renal ischemia/reperfusion (I/R) injury is an inevitable tissue injury caused by blood supply return to the cold organ during kidney transplantation. I/R injury is a major cause of acute kidney injury and delayed graft function[1]. To overcome this problem, cell death of the proximal tubule cell, which is known to be the main target of renal I/R injury[2], should be blocked.

It is widely accepted that electromagnetic field (EMF) exposure exerts various biological effects on cell proliferation, differentiation, cell cycle, apoptosis, DNA replication, and gene expression[3]. In addition, it has been reported that EMF exerts protective effects on various cell types[4]. However, no investigations of EMF's effects on renal I/R injury have been conducted to date.

Therefore, in this study, we examined the protective effects of EMF on the renal proximal tubular cell, which is among the cell types most sensitive to I/R injury and the main site of acute kidney injury[2], using a previously established *in vitro* hypoxia/reoxygenation (H/R) injury model[5].

Renal I/R injury was induced *in vitro* according to the previous study[5]. Briefly, the human renal proximal tubular cell line HK-2 cells were cultured in DMEM/F12 medium, supplemented with 10% heat-inactivated FBS, antibiotic-antimycotic and insulin-transferrin-selenium at 37 °C in a 5% CO_2 atmosphere. Before experimental intervention, confluent cultured cells were serum-starved for 24 h in DMEM/F12 with antibiotic-antimycotic, 0.1% FBS and insulin-transferrin-selenium. Then, serum-starved cells were exposed to 24 h of hypoxia (1% O_2) followed by 12 h of reoxygenation (20% O_2).

The EMF generated by two coils arranged in a Helmholtz configuration oriented to produce a vertical magnetic field with parameters as following: axial symmetry (2D), diameters of two coils (inner diameter=15 cm, outer diameter=26 cm), the distance between two coils (18 cm), coil diameter (AWG), and number of loops (N=1000) (Figure 1). The system was placed in a Heracell gas addition incubator (Heraeus Instruments GmbH, Hanau, Germany) which is capable of controlling O_2. The cells were exposed to different conditions with continuous sinusoidal extremely low frequency of EMF [(10-100) Hz/(0.01-1) mT] by using AC power source (PCR-1000L, Kikusui, Japan). The cell culture dishes were maintained at the center of a uniform field area. A thermometric probe placed inside and outside the EMF generator revealed no significant temperature difference between culture media of exposed or unexposed cells.

The cell viability was determined by trypan blue exclusion assay. For trypan blue exclusion assay, 10 µL aliquots of cells were mixed with 0.4% trypan blue (ACROS Organics, New Jersey, USA) in PBS and 10 µL of mixture was pipetted onto side of a hemocytometer chamber (Paul Marienfield GmbH & Co.KG, Germany). The hemocytometer was viewed

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under an inverted microscope (Eclipse TS100-F, Nikon, Tokyo, Japan), and the total number of cells (intact and damaged) was counted. The viability percentage was obtained by multiplying the fraction of the number of viable cells over the total number of cells by 100.

The intracellular ROS was evaluated by using cell-permeable probe 2, 7'-dichlorofluorscein-diacetate (DCFH-DA). Inside cells, DCFH-DA was cleaved by esterase forming DCFH, which was in non-fluorescent form and was oxidized to fluorescent compound DCF by ROS. Following the exposure to 20% (negative control) or 1% O_2, the cells were loaded with 10 μmol/L DCFH-DA for 60 min at 37 °C. The fluorescence signals (Ex 485 nm, Em 535 nm) were measured with Victor X4 multilabel plate reader (PerkinElmer, Massachusetts, USA).

Statistical significance of the differences was assessed by one-way ANOVA with Tukey's HSD post hoc test by using statistical software SPSS 12.0 for Windows. Values are expressed as the means±standard deviation (SD). Differences were considered significant when $P<0.05$.

We investigated the protective effects of extremely low frequency EMF on the cell survival of the human renal proximal tubular cell line, HK-2, using an *in vitro* H/R model. HK-2 cells were cultured in hypoxia for 24 h followed by 12 h-reoxygenation, then the cell survival was measured. The survival rate of the cells cultured under this condition declined by approximately 45% compared with control cells (cultured in 20% O_2 without a hypoxia period). However, the survival rate of cells exposed to 10 Hz/1 mT EMF during cell culture under the same H/R condition was about 15% higher than that in equivalent non-exposed cells (Figure 2A).

To determine whether this protective effect depends on EMF frequency, we evaluated the effects of three different EMF frequencies (10, 50, or 100 Hz) on cell survival. As shown in Figure 2B, 50 or 100 Hz/1 mT EMF exposure did not exert any protective effect, while 10 Hz/1 mT EMF did, indicating a frequency-specific protective effect. Then we tested the intensity specificity by using three different EMF intensities (0.01, 0.1, or 1 mT). As shown in Figure 2C, 0.01 or 0.1 mT/10 Hz EMF exposure did not exert any protective effect, while 1 mT/10 Hz EMF exposure protected the cells. These results suggest that the protection of HK-2 cells from H/R-induced cell death requires a specific frequency (10 Hz) and intensity (1 mT) of EMF.

We examined intracellular ROS levels of HK-2 since it has been reported that H/R-induced cell death is associated with increased levels of intracellular ROS[6]. The intracellular ROS levels of the cells cultured under H/R condition increased to approximately 190% of the control levels while those of the cells exposed to 10 Hz/1 mT EMF became about 145% of the control (Figure 3A). These results suggest that the protective effect of EMF on H/R-induced cell death is associated with the reduction of intracellular ROS.

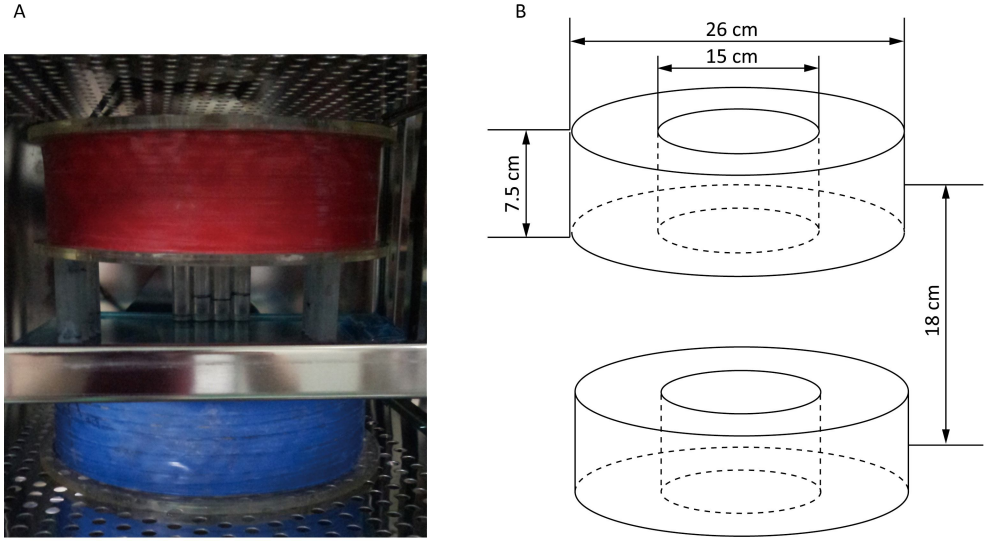

Figure 1. A photograph of a helmholtz coil installed in the incubator (A) and a schematic diagram of coils with specification (B).

To determine whether EMF-induced ROS reduction depends on EMF frequency, we examined the intracellular ROS levels following exposure to three different EMF frequencies (10, 50, or 100 Hz). As shown in Figure 3B, 50 or 100 Hz/1 mT EMF exposure did not cause any reduction of ROS, while 10 Hz/1 mT EMF exposure did, indicating frequency-specific ROS reduction. Then we tested the intensity dependence of the EMF effect by applying different EMF intensities (0.01, 0.1, or 1 mT) and measuring the intracellular ROS. As shown in Figure 3C, the exposure to 0.01 or 0.1 mT/10 Hz EMF did not have any effect on ROS levels, while 1 mT/10 Hz EMF exposure decreased ROS. These results suggest that the reduction of intracellular ROS production by EMF exposure depends on the specific frequency and intensity.

The major findings of this study are: 1) the exposure to 10 Hz/1 mT EMF inhibits H/R injury-induced cell death of the human renal proximal tubular cell line, HK-2; 2) One of the protective mechanisms is the reduction of intracellular ROS production in response to EMF exposure.

Both basic and clinical studies suggest that exacerbation of I/R injury is mainly mediated by the rapid production of ROS[6], which is considered to be the main cause of cell death. Accordingly, the suppression of ROS production during the I/R process would be an important treatment for the protection of renal cells. *In vitro* studies[7-8] and *in vivo* studies using rats[9] revealed that EMF exposure increases cellular ROS generation, which contradicts the results of the present study. This discrepancy might

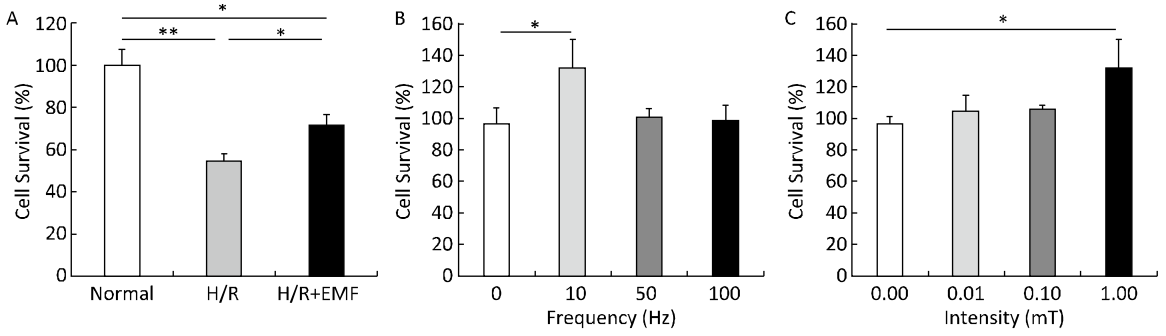

Figure 2. EMF protects HK-2 from H/R-induced cytotoxicity. (A) Cells were cultured under normal or H/R conditions with or without EMF exposure (10 Hz/1 mT). (B) Cells were cultured under H/R conditions with 1 mT EMF at various frequencies (10, 50, and 100 Hz). (C) Cells were cultured under H/R conditions with 10 Hz EMF at different intensities (0.01, 0.1, and 1 mT). Cell survival was determined by microscopic examination of cells stained with trypan blue. Zero on the X-axis designates no EMF exposure. Bar graphs show relative cell survival±SD. $^{*}P<0.05$, $^{**}P<0.001$.

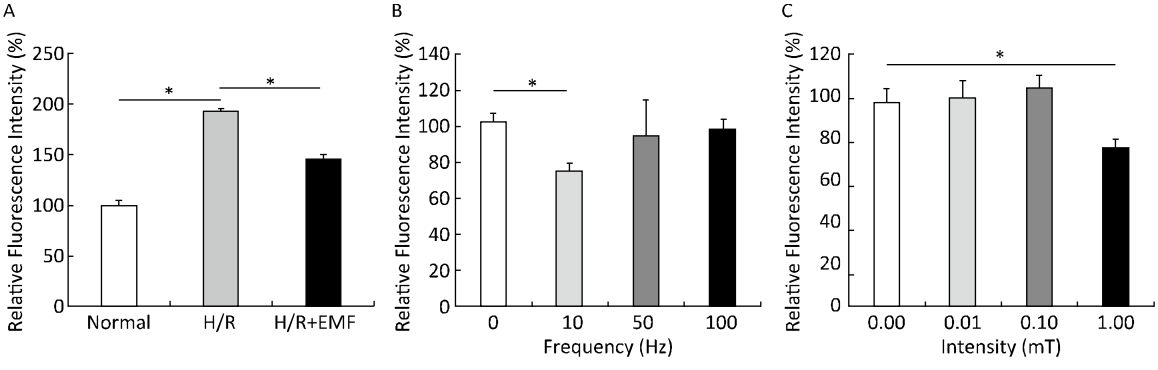

Figure 3. EMF reduces H/R-induced intracellular ROS production of HK-2. (A) Cells were cultured under normal or H/R conditions with or without EMF exposure (10 Hz/1 mT). (B) Cells were cultured under H/R conditions with 1 mT EMF at various frequencies (10, 50, and 100 Hz). (C) Cells were cultured under H/R conditions with 10 Hz EMF at various intensities (0.01, 0.1, and 1 mT). Intracellular ROS levels were measured by fluorometry. Zero on the X-axis designates no EMF exposure. Bar graphs show relative intracellular ROS levels±SD. $^{*}P<0.01$.

be due to differences in the experimental conditions used. The previous studies did not use H/R condition and used 50 or 60 Hz EMF, while we used H/R condition, which has been known to induce cellular ROS production, and different EMF frequency of 10 Hz in our study. The mechanism of the suppressive effect of EMF on H/R-induced ROS production in our study is unclear. In a previous study, moderate decreases in cellular ROS levels were detected in EMF-exposed neutrophils, suggesting that the biological activities of some ROS-producing enzymes might be decreased[10]. In contrast, the activities of some ROS-scavenging enzymes such as peroxidase or glutathione-S-t ransferase were up-regulated in response to EMF exposure[10].

In summary, the present study demonstrated that EMF exposure can inhibit H/R injury-induced cell death of human renal proximal tubular cells through the suppression of intracellular ROS production, and that this protective effect depends on the frequency and the intensity of EMF. Since EMF exposure is a physical and non-invasive treatment without the risk of complicated drug interactions and drug side effects, it might be clinically useful to ameliorate renal I/R injury in the near future if the protective effect of EMF observed in the present study is further confirmed by an animal study.

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