Extremely Low Frequency Magnetic Fields Do Not Elicit Oxidative Stress in MCF10A Cells

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ELF-MF/Oxidative stress/MCF10A cells.

The aim of this study was to determine whether extremely low frequency magnetic fields (ELF-MF) could affect intracellular reactive oxygen species (ROS) levels and antioxidant enzyme activity. After MCF10A human breast epithelial cells were exposed to 1 mT of 60 Hz ELF-MF for 4 hours, intracellular ROS level, superoxide dismutase (SOD) activity, and reduced to oxidized glutathione (GSH/GSSG) ratio were measured. The cells exposed to ELF-MF did not evidence statistically significant changes in the above-mentioned biological parameters as compared to either the incubator controls or sham-exposed cells. By way of contrast, the IR-exposed cells exhibited marked changes in ROS level, SOD activity, and GSH/GSSG ratio. When we assessed morphological changes and senescence-associated beta-galactosidase (SA-β-Gal) activity, only the IR-exposed cells were positive. According to our results, it could be concluded that ELF-MF has no effect on intracellular ROS level, SOD activity, and GSH/GSSG ratio under our exposure condition.

INTRODUCTION

Extremely low frequency magnetic fields (ELF-MF), which are conventionally defined as those produced by alternating current at between 30 and 300 Hz, are generated by power distribution networks, industrial machinery, and electric appliances. General public is exposed continuously to ELF-MF on a daily basis in industrialized nations.[1] Therefore, the biological effects of ELF-MF have been a subject of investigation for a long period of time. A great deal of research has been focused on the possible relationship between ELF-MF and variety of biological processes.[2–5] ELF-MF exposure has been previously suggested its association with increased incidence of certain tumor types, particularly leukemia and brain cancer.[6–8] Those epidemiological data have been explained by the radical pair mechanism.[9]

It has been suggested that 50 Hz magnetic fields may affect the formation of superoxide radicals.[10] It has been also reported that ELF-MF exposure can modulate redox related protein expression or activity, finally leading to ROS generation.[11,12] Excessive ROS generation induces DNA damage, which may lead to carcinogenesis, implicating that ELF-MF could act as a cancer initiator in different cell types and under different experimental conditions.[13,14] By way of contrast, antioxidant enzyme activities and protein expressions which were involved in regulation of cellular redox state have been appeared not to be affected by ELF-MF.[15,16] Absence of genotoxicity in cells which were exposed to ELF-MF proved that excessive oxidative stress may not be induced due to ELF-MF exposure.[17] Since the relationship between exposure to ELF-MF and formation of free radicals in the living cells remains ambiguous as mentioned above, further elucidation is still needed.

ROS is a product of normal aerobic metabolism and are generally produced in the mitochondria. The ROS generation processes also play a decisive role in important biological processes, such as inflammation, cell division, differentiation, apoptosis, and cellular senescence. Currently, it has been demonstrated that ROS and ROS-mediated biochemical modifications perform important functions not only in the carcinogenic process, but also in the physiological aging progression which is strictly associated with radical-mediated oxidative damage.[18] ROS is a known causative factor in the oxidative damage of cellular structures and macromolecules such as lipids, proteins, and nucleic acids.[19] Cells harbor

large quantities of antioxidants to prevent or repair the damage caused by ROS, and to regulate redox-sensitive signaling pathways. Intracellular ROS concentrations are under the control of antioxidant enzymes. The intrinsic antioxidant enzyme system has been identified as a defense mechanism that protects cells against oxidative injury.[20] Three primary antioxidant enzymes which are necessary for life in all oxygen-metabolizing cells are superoxide dismutase (SOD), catalase (CAT) and the substrate-specific peroxidase, glutathione peroxidase (GPx).[21]

Free radical theory was recently proposed as one of the most important and possible biological mechanisms of ELF-MF activity. The World Health Organization (WHO) Research Agenda (2007) classified the *in vitro* studies regarding the evaluation of reactive oxygen species into a high-priority line of inquiry in the research field of biological effect of EMF. The principal objective of this study was to elucidate whether ELF-MF exposure could elicit oxidative stress in cultured human breast epithelial MCF10A cells. We determined intracellular ROS level, antioxidant enzyme activity, GSH/GSSG ratio, and cellular senescence in ELF-MF exposed cells.
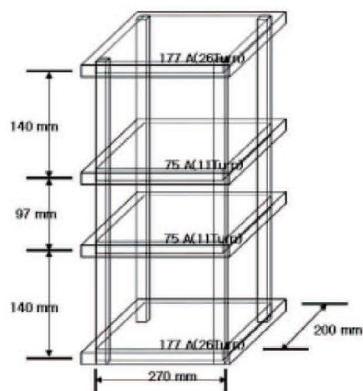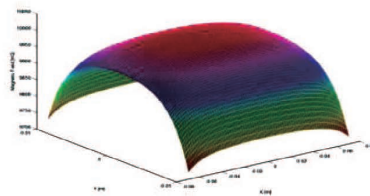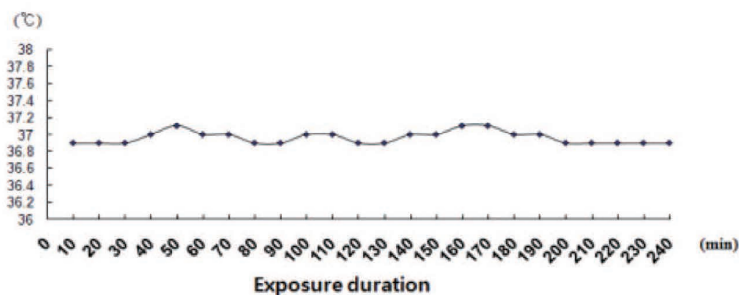

Fig. 1. ELF-MF exposure system. **A**: Diagram and photo of exposure system. **B**: Power generator. **C**: The voltage fluctuation rate and harmonic rate of power quality. **D**: Total exposure system including incubator. **E**: Temperature variation during experimental period in exposure system.

MATERIALS AND METHODS

Cell cultures

MCF10A human breast epithelial cells was purchased from the American Type Culture Collection (ATCC, Manassas, USA) and grown in DMEM/F12 medium with L-glutamine (Invitrogen, Carlsbad, USA) supplemented with 10% heat-inactivated horse serum (Invitrogen, Carlsbad, USA), 21 ng/mL of EGF (Peprotech, NJ, USA), 0.525 µg/mL of Hydrocortizone (Sigma, St. Louis, USA), 0.05 µg/mL of CholeraToxin (Sigma, St. Louis, USA), 11.2 µg/mL of Insulin (Sigma, St. Louis, USA), 100 mg/mL of streptomycin (WelGENE, Daegu, Korea), and 100 U/mL of penicillin (WelGENE, Daegu, Korea) in a 5 % CO_2 incubator at 37°C.

Extremely low frequency magnetic fields (ELF-MF) exposure system

ELF-MF generation equipment was designed and constructed by Korea Electrotechnology Research Institute as shown in Fig. 1A. ELF-MF monitoring was conducted under observation of the current injected to exposure system, since ELF-MF is proportional to injected current. The field generator consists of four square-shaped coils and one cage with three testing floor (top, middle, and bottom floor). Power supply is shown in Fig. 1B. The voltage fluctuation rate and harmonic rate of power quality using power amplifier was under 1%. Figure 1C shows calculated magnetic field distribution at the middle floor in exposure system. Fixing the magnetic field at the center of the middle floor at 1 mT. The fields at various points were measured under the condition of uniformity of ELF-MF exposure system is shown in Table 1. The spatial variation of magnetic field was under 3%. This strongly dem-

Table 1. Uniformity of MF exposure system.

Location	1 mT Reference	Uniformity
Top Floor	1.016	
	1.013	
	1.015	
	1.019	
	1.015	
Middle Floor	1.018	
	1.019	
	1.021	1%–2.6%
	1.008	
Low Floor	1.013	
	1.012	
	1.010	
	1.019	
	1.026	

onstrates that the field generator is good for a small-sized *in vitro* study. Total ELF-MF exposure system including incubator is demonstrated in Fig. 1D. The temperature of incubator was maintained 37 ± 0.3°C during exposure period using water-jet cooling system as shown in Fig. 1E. Magnetic field shielding system using ferrite material was adopted to shield strong magnetic field in outer of ELF-MF exposure system.

Exposure to ELF-MF and γ-radiation

ELF-MF exposure system was warmed up for 15 min to equilibrate it prior to ELF-MF exposure. And then, cells in 100 mm Petri dish were exposed to 60 Hz at 1 mT for 4 h. Maximum MF of our exposure system (1 mT, ~ two folds of the occupational reference level of 60 Hz magnetic field which was guided by International Commission on Non-ionizing Radiation Proteiction (ICNIRP)) was chosen for this study.[22] After exposure, the cells were immediately transferred to a cell culture incubator and kept for the post-exposure time (10, 24, or 48 h) before harvesting the cells. For the sham exposure, the cells were kept in the ELF-MF exposure device, but were not exposed to ELF-MF. The temperature of the exposure device was monitored throughout the exposure period in the sham-exposed and the ELF-MF exposed groups. For the positive control group, cells were exposed to 0.5, 2, or 4 Gy of γ-radiation from a ^{137}Cs gamma ray source (Atomic Energy of Canada, Mississauga, Canada) at a dose rate of 3.81 Gy/min.

Measurement of SOD activity

SOD activities in the MCF10A cells were measured using a SOD assay kit-WST (Dojindo Molecular Technologies, Kumamoto, Japan), according to the manufacturer's instructions. Briefly, Cells were harvested and suspensed in lysis buffer (100 µL of ice-cold 50 mmol/L of D-mannitol, 2 mmol/L of Tris-HCl, pH 7.4, 0.1% of Triton X-100) and sonicated 30 seconds for 8–10 times until the solution was cleared. After 15 minutes, the sonicated sample at $1,000 \times g$ for 15 min at 4°C, the supernatant was transferred to a new tube and diluted with dilution buffer to prepare a sample solution. The SOD activity was expressed as inhibition rate percent per the manufacturer's instructions.

Measurement of reduced and oxidized glutathione level

A Biooxytech GSH/GSSG-412 kit (Oxis International, Portland, USA) was used to measure reduced and oxidized glutathione (GSH/GSSG). To measure GSH/GSSG ratio, cells were harvested and suspensed with lysis buffer which were described above. Lysates mixed gently with or without 33 mmol/L 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP), a scavenger of reduced glutathione (GSH). And then, six volumes of 5% metaphosphoric acid added to the sample solutions. The sample solutions were centrifuged at $1,000 \times g$ for 10 min. For GSH estimation, 20 µL supernatant was mixed with 1,220 mL assay buffer (100

mmol/L NaPO$_4$ and 5 mmol/L EDTA, pH 7.5) For GSSG estimation, 50 µL supernatant was mixed with 700 µL GSSG buffer (100 mmol/L NaPO$_4$ and 5 mmol/L EDTA, pH 10.05). The samples were mixed with 1.262 mmol/L 5,5-dithiobis-(2-nitrobenzoic acid) and 15 units/mL GSH reductase. The mixture was incubated at room temperature for 5 minutes, and the absorbance was recorded at 412 nm for 3 minutes after the addition of NADPH.

Flow cytometric analysis of reactive oxygen species

The intracellular ROS level was assessed using the 2', 7'-dichloro-fluorescein diacetated compound (DCF-DA). DCF-DA (Sigma, St. Louis, USA) was added directly to the culture medium at a final concentration 10 µM. Cells were then incubated at 37°C for 20 minutes, washed once in PBS (WelGENE, Daegu, Korea) and immediately analyzed using a FACScan flow cytometer (Becton Dickinson, NJ, USA) with excitation and emission at 490 and 530 nm.

Statistical analysis

All data were expressed as mean ± standard deviation (M ± SD) from three or five independent experiments in duplicate. Differences between control and exposed groups were analyzed using a Student's t-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of ELF-MF exposure on intracellular ROS level

We evaluated the effects of ELF-MF on intracellular ROS levels using DCF-DA. Exposure to 60 Hz ELF-MF at 1 mT for 4 hours did not affect intracellular ROS levels relative to the sham-exposed group (Fig. 2B). On the contrary, IR exposure increased intracellular ROS levels in a dose-dependent manner compared to the controls in MCF10A cells at each time point (Fig. 2A). These results were analyzed statistically

and values of $p < 0.05$ were considered statistically significant.

Effect of ELF-MF exposure on SOD activity

Next, we conducted SOD activity assays, as SOD has been recognized as an antioxidant enzyme that elicits a primary defense response when cells are challenged by oxidative stress. As shown in Fig. 3B, exposure to 60 Hz ELF-MF at 1 mT did not significantly alter SOD activity in a time- or dose-dependent manner, relative to the sham-exposed group. However, SOD activity was significantly increased in the IR-exposed groups (Fig. 3A).

### Effect of ELF-MF exposure on reduced/oxidized glutathione levels

Reduced glutathione (GSH) level is noted in all cellular compartments and GSH functions as a cell protectant, specifically as a radical scavenger via a cycling redox process. We measured reduced to oxidized glutathione ratio (GSH/GSSG), because oxidized glutathione accumulates in cells under oxidative stress challenge conditions.[20] When we measured GSH/GSSG ratio following exposure of the cells to ELF-MF, the ratio was reduced in the IR-exposed groups as shown in Fig. 4A. By way of contrast, GSH/GSSG ratio evidenced no statistical difference between the ELF-MF-exposed and sham-exposed groups (Fig. 4B).

### Effect of ELF-MF on cellular morphology and SA-β-Gal activity

Finally, we evaluated the effects of ELF-MF on senescence-associated phenotypic changes. IR-exposed cells exhibited senescence-specific large and flat cellular morphology and stained positively for SA-β-Gal staining. Such senescence-associated phenotypic changes were detected in dose- and time-dependent manners in the IR-exposed groups (Fig. 5A). Quantitative analysis of SA-β-Gal positivity is shown in Fig. 5B. However, 60 Hz ELF-MF at 1 mT affected neither cellular
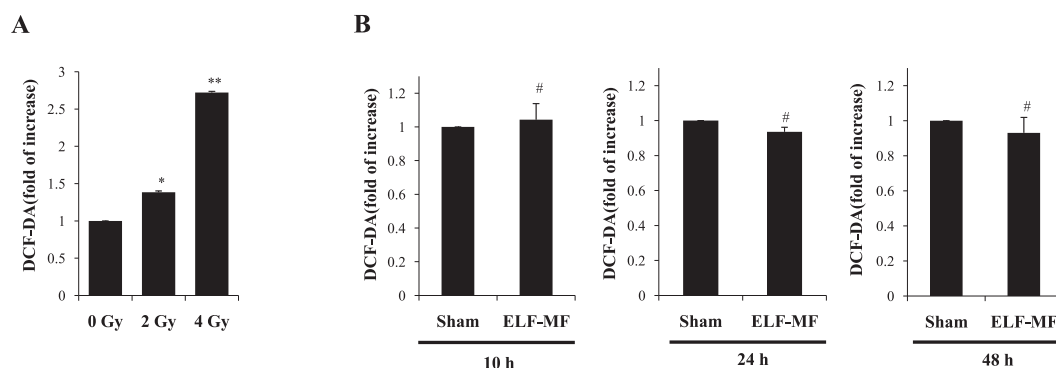
Fig. 2. Effect of ELF-MF on intracellular ROS levels in MCF10A cells. **A**: Effect of IR on intracellular ROS level in MCF10A cells exposed to 0, 2, and 4 Gy doses of IR. ROS levels were evaluated 48 h after IR exposure. **B**: Effect of ELF-MF on intracellular ROS level in MCF10A cells. Cells were exposed continuously to 1 mT for 4 h and ROS level was analyzed at 10, 24, and 48 h after recovery times via flow cytometry. Bars show the mean values and standard deviations (M ± SD) of five independent experiments in duplicate. Significantly different: *$p < 0.05$ versus 0 Gy, **$p < 0.01$ versus 0 Gy, insignificantly different: #$p > 0.05$ versus sham.

A   **B**
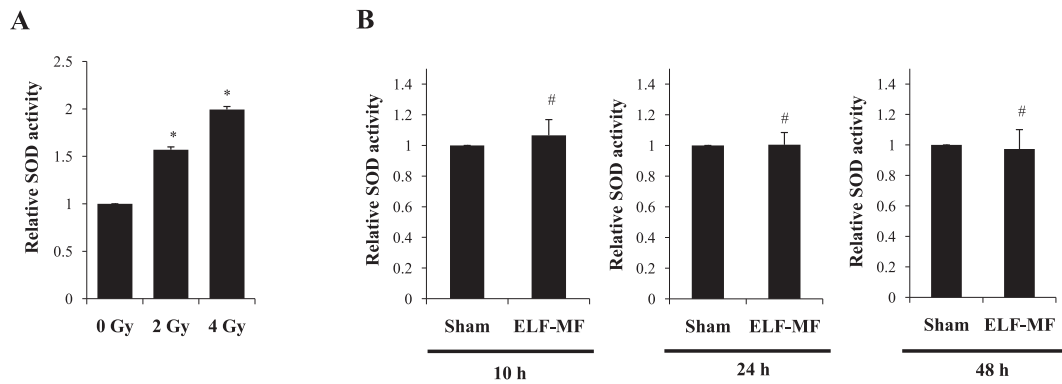
Fig. 3. Effect of ELF-MF on SOD activity in MCF10A cells. **A**: SOD activity in MCF10A cells exposed to 0, 2, and 4 Gy doses of IR. **B**: Effect of ELF-MF on SOD activity in MCF10A cells. Cells were exposed continuously to 1 mT for 4 h and SOD activity was measured at 10, 24, and 48 h after recovery times. Bars show the mean values and standard deviations (M ± SD) of five independent experiments in duplicate. Significantly different: *p < 0.05 versus 0 Gy, insignificantly different: #p > 0.05 versus sham.

**A**   **B**
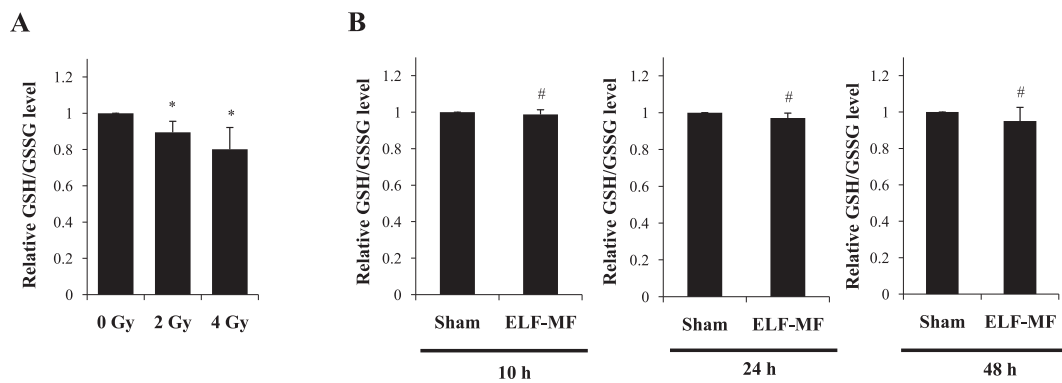
Fig. 4. Effect of ELF-MF on GSH/GSSG level in MCF10A cells. **A**: Analysis of GSH/GSSG levels in MCF10A cells exposed to 0, 2, and 4 Gy doses of IR. **B**: Analysis of GSH/GSSG levels in MCF10A cells which were exposed to 60 Hz ELF-MF at 1 mT for 4 h. GSH/GSSG levels was assesssed at 10, 24, and 48 h after recovery times. Bars show the mean values and standard deviations (M ± SD) of five independent experiments in duplicate. Significantly different: *p < 0.05 versus 0 Gy, insignificantly different: #p > 0.05 versus sham.

morphology nor SA-β-Gal activity (Fig. 5C). When SA-β-Gal positivity was assessed in sham and ELF-MF exposed groups, there was no statistical difference (data not shown).

DISCUSSION

ROS including oxygen free radicals and other activated oxygen species have been previously implicated in a variety of physiological and pathological conditions: namely, the aging process, inflammatory injury, atherosclerosis, and arthritis.[23] Intracellular ROS concentration was determined according to the balance between the ROS production via mitochondrial or nonmitochondrial pathways and clearance rates by various antioxidant compounds or antioxidant enzymes. As increased levels of intracellular ROS could be associated directly with DNA damage and carcinogenesis, it is important to assess and elucidate the effects of ELF-MF on oxidative stress.

Several studies have previously demonstrated that ELF-MF could affect ROS production, and may function as an oxidative stress. It has linked the 50 Hz ELF-MF capacity for the activation of immune-relevant cells to the free radical-based physiological changes.[24,25] Moreover, it was reviewed that ELF-MF exposure resulted in excessive free radical production through immune cell activation, in turn leading to genotoxic event.[26] On the other hand, Frahm et al.[27] reported that 50 Hz ELF-MF elevated phagocytic activity and intracellular ROS level in the absence of any genotoxic effects after 24 h exposure at 1 mT. It has been also reported that 50 Hz ELF-MF exposure reduced cell tolerance towards oxidative attack without any genotoxic and cytotosic effect in neuroblastoma cells.[28] Fiorani et al.[29] proved that 50 Hz MF (0.2–0.5 mT) could be able to potentiate the cellular damage induced by oxidizing agents in rabbit red blood cells. Other study showed that ELF-MF did not cause oxidative damage in cellular DNA in human placental cotyle-

A

B

C
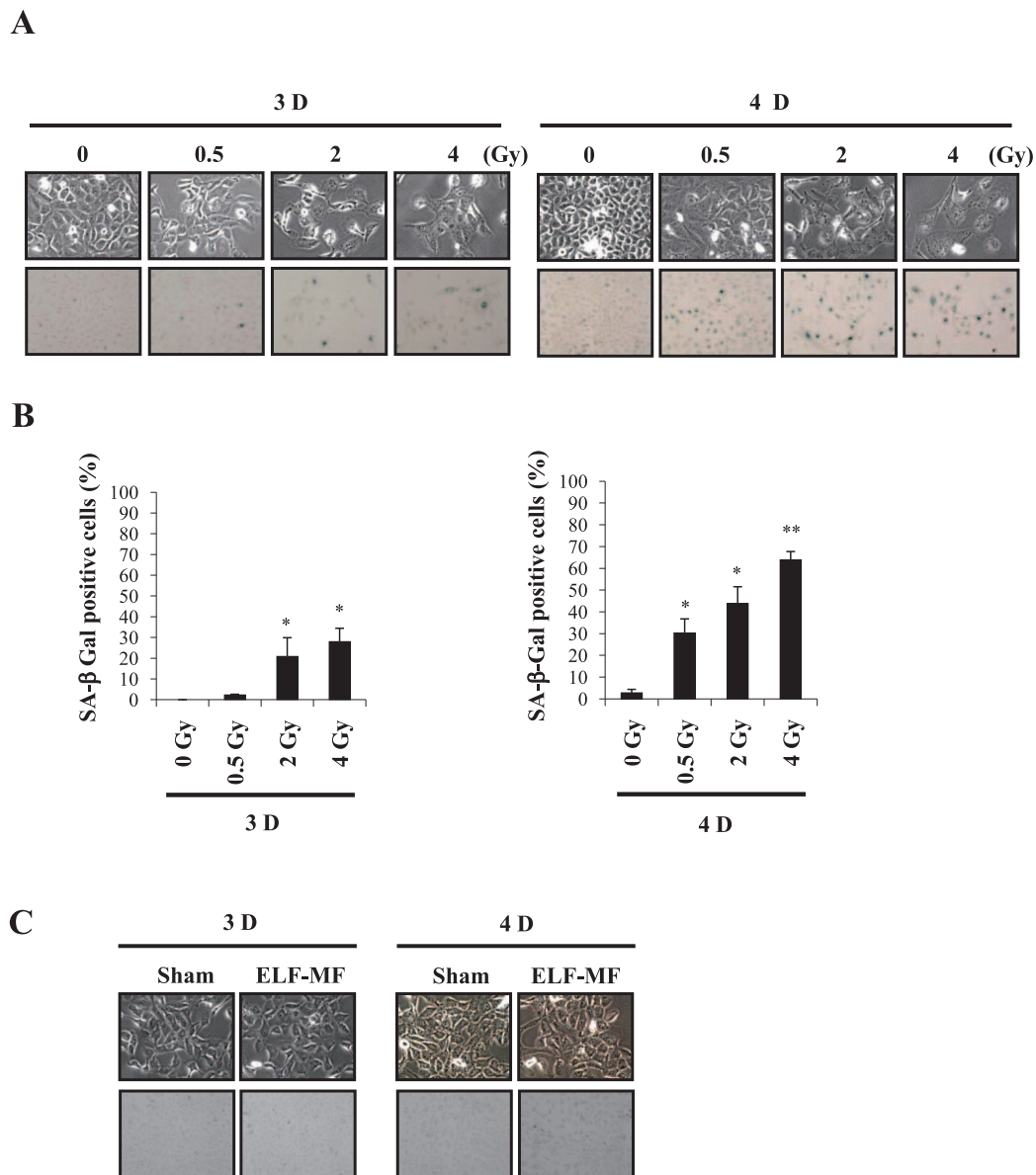
Fig. 5. Effect of ELF-MF on cellular morphology and SA-β-Gal activity. **A**: Cellular morphology and SA-β-Gal activity in MCF10A cells exposed to 0.5, 2 and 4 Gy doses of IR. **B**: Quantitative analysis of SA-β-Gal positivity in IR-exposed cells. **C**: Cellular morphology and SA-β-Gal activity in MCF10A cells exposed to 60 Hz ELF-MF at 1 mT for 4 h. Cellular morphology and SA-β-Gal activity were assessed at 3 and 4 days after exposure, via phase-contrast microscopy. Bars show the mean values and standard deviations (M ± SD) of three independent experiments. Significantly different: *$p < 0.05$ versus 0 Gy, **$p < 0.01$ versus 0 Gy.

dons *in vitro* after exposure to low dose 50 Hz ELF-MF (2 and 5 mT for 3 h).[30] Luukkonen *et al.*[31] recently found that pre-exposure to 50 Hz MF enhanced menadione-induced DNA damage, DNA repair rate, and micronucleus formation in human SH-SY5Y neuroblastoma cells. However, they found no effect of ELF-MF alone exposure on DNA damage and repair rate. As menadione is a radical-inducing agent, authors described that MF might affect free radical processes, as suggested by the radical pair mechanism. Brocklehurst and McLauchlan[9] have been suggested, although the radical

pair mechanism has been hypothsized as a possible route whereby MF of environmental strength might affect a biological system, freely diffusing pairs of radicals are unlikely to produce significant effects in biological system. In the present study, we assessed ELF-MF influence on the ROS level and found that exposure to 60 Hz ELF-MF at 1 mT did not affect intracellular concentration of ROS in MCF10A cells.

Cellular macromolecules, i.e, DNA, protein, and lipid are the most susceptible molecules to free radical attack. Therefore, living organisms harbor a variety of defense systems

including antioxidant vitamins, SOD, CAT, GSH, and glutathione peroxidase (GSH-Px) to protect cells against oxidative stress. Among them, GSH/GSSG, one of the best-characterized antioxidant defense mechanisms, is the most rapid and abundant weapon against ROS. Its protective activity is predicated on the oxidation of the thiol group of its cysteine residue with the formation of GSSG, which is, in turn, catalytically reduced back to the thiol form (GSH) by glutathione reductase (GSSG-Rd).[32] Decrease of intracellular GSH level was reported as the result of 50 Hz ELF-MF simultaneous exposure at 0.5 mT for 90 min with the Fe(II)/ascorbate system in rabbit erythrocytes.[28] It has been suggested that GSH could play an important role in the antioxidant defense mechanism, due to finding of a positive correlation between reduced glutathione (GSH) and ROS levels after 50 Hz ELF-MF exposure at 1 mT for 7 days in rat cortical neurons.[33] Another antioxidant enzyme, SOD, performs a crucial role in the system, protecting cells against destructive free radical activity. This antioxidant defense system could be deteriorated by a 50 Hz ELF-MF at 8.8–84 μT MF strength and 20–133 V/m electric field strength, which might lead to oxidative stress in human red blood cells.[34] Zwirska-Korczala *et al.* demonstrated that 180–195 Hz ELF-MF influences enzyme activities of SOD, catalase, GSH-Px, and GSSG-Rd in 3T3-L1 preadipocytes after ELF-MF exposure (120 μT, 36 min daily for 2 days).[35] It was also demonstrated that SOD activity was increased as result of 60 Hz ELF-MF exposure at 1.2 mT for 3 h in mouse brain, suggesting that such increase may be reflective of a compensatory reaction against oxidative stress.[36] Our study employed assessments of the GSH/GSSG ratio and SOD activity, since both of which reflect the strength of the antioxidant defense system and are also indicatives of oxidative stress. We found that exposure to 60 Hz ELF-MF at 1 mT affected no significant changes in GSH/GSSG and SOD activity. Intracellular ROS (superoxide and hydrogen peroxide) are produced from the mitochondrial electron transport chain and enzymatic reactions. Superoxide is converted to hydrogen peroxide by SOD, and catalase neutralizes hydrogen peroxide to water.[37] Since we measured intracellular ROS level using DCF-DA and SOD activity, it would be interesting if intracellular superoxide level was further assessed using dihydroethidium (DHE) probe. In addition, since we monitored ELF-MF alone effect on oxidative stress, it would be also valuable to elucidate whether EMF-MF could potentiate oxidative stress which is induced oxidative stimuli in various cell lines. Therefore, more extensive studies to elucidate whether MF might induce genetic damage and/or affect a biological system via generation of oxidative stress are necessary.

Cellular senescence is irreversible cell cycle arrest, which is activated in response to variety of cellular stresses, specifically to the oxidative stress.[38,39] Actually, the radical theory is concerned in the field of cellular senescence by a growing body of evidence that damaged cellular function due to the increase of free radical could be associated with a number of senescence-related phenotypes.[22,38] Senescent cells undergo morphological changes with flat and large size and most commonly used senescence biomarker is SA-β-Gal positivity.[40,41] Therefore, we evaluated effect of ELF-MF on cellular senescence via the examination of cellular morphology and SA-β-Gal activity. We could not observe any detectable changes in cellular morphology and SA-β-Gal activity after ELF-MF exposure.

Collectively, the results of this study demonstrated that 1 mT ELF-MF exposure did not affect intracellular ROS level, SOD enzyme activity, GSH/GSSG ratio, and phenotypical changes of cellular senescence in MCF10A cells under our exposure condition. The publications regarding the biological impact of ELF-MF have been accumulated, however, contradictory and ambiguoucity are still existed due to the variations of biological systems and endpoints. In addition, some papers have not been considered temperature variation in *in vitro* exposure system during the ELF-MF exposure. We suggest that future experiments have to be performed under the carefully controlled exposure conditions and with proper positive and negative controls to get closer to final conclusion regarding concerns about harmful effect of ELF-MF exposure.

ACKNOWLEDGEMENTS

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