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Effects of 940 MHz EMF on Bioluminescence and Oxidative Response of Stable Luciferase Producing HEK Cells

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Abstract

The effects of mobile phone frequency electromagnetic field (RF-EMF, 940 MHz) on a stable cell line (HEK293T) harbouring firefly luciferase gene were evaluated. A waveguide exposure system with 1W input power provided the mean specific absorption rate of ≈ 0.09 W/kg in 35 mm Petri dishes. The effects of exposure duration (15, 30, 45, 60 and 90 min) were investigated on luciferase activity and oxidative response elements. Endogenous luciferase activity reduced after 30 and 45 min continuous exposures. While, after 60 min, the exposed cell lysate showed higher luciferase activity compared with the non-exposed control. Reactive oxygen species (ROS) generation was highest in the 30 min exposed cells as studied by DCFH-DA fluorescence.

The observed boost in ROS was then followed by a sharp rise in catalase (CAT) and superoxide dismutase (SOD) activity and elevation of glutathione (GSH) during the 45 min exposure. Decrease in lipid peroxidation (malondialdehyde, MDA) was meaningful for the 45 and 60 min exposed cells. Therefore, it appears that an increase in the activity of luciferase after 60 min of continuous exposure could be associated with a decrease in ROS level caused by activation of oxidative response. This ability in cells to overocme the oxidative stress and compensate the luciferase activity could also be responsible for the adaptive response mechanism detected in ionizing radiation studies with RF-EMF pre-treatments.

Key words: 940 MHz, EMF, Luciferase, HEK293T, Oxidative stress

Introduction

Research around the effects of electromagnetic fields (EMF) in biological systems have included a wide variety of studies. Such studies have ranged from inter and intra cellular interactions[1] to finding relationships with cognition, apoptosis,[2] cancer,[3] neurodegenerative diseases[4] and possible beneficial issues.[5] Health endpoints reported to be connected with radiofrequency EMF are childhood leukemia, brain tumors, genotoxicity, neurodegeneration, immune system deregulation, allergic responses, infertility and some cardiovascular diseases.[6]

The global system for mobile communication works in different ranges of frequencies across the countries and consists of uplink (i.e., transmission from handset to the base station) and downlink (transmission from the base station to handset). Near-field exposure occurs while using communication devices such as mobile or cordless phones close to the body (< 10 to 30 cm).[7] Close to body sources are generally responsible for localized exposure (e.g. in the head area) and the exposure is mostly limited to short time periods (mobile phone), while far-field exposures are usually whole body and long-term (base station).[8]

Different mechanisms have been proposed to explain the observed cellular effects of electromagnetic fields.[9] It has been claimed that possible effects could be mediated by indirect mechanisms such as microthermal processes, generation of reactive oxygen species (ROS), disturbance of DNA-repair processes and interacting with signalling pathways.[10]

George *et al.* indicated that there is a difference between the heating that is provided by immersion in a water bath (conduction) and that induced by microwaves (which produces a higher heating rate).[11] Small variations of temperature are inevitable in EMF studies. However, a

significant elevation of temperature in cell culture media has been observed at high specific absorption rate (SAR) levels. Heat shock responses are very sensitive to the increase in temperature. Consequently, heat shock response might not be a good indicator to describe non-thermal effects of RF-EMFs.[12] In general, exposure bellow ICNRP guidelines (SAR of 2 W/kg for local exposure in the frequency of interest in this study) will decrease the risk of interference between non-thermal and relevant heating effects. However, heating may occur at any level of exposure and there is no threshold.

The formation of free radicals and the processes by which they are controlled and scavenged are critical determinants of cellular injury and is highly relevant to processes such as aging, cancer and reproductive outcomes.[13] The defense mechanisms against oxidative damage of ROS include superoxide dismutase (SOD) and catalase (CAT) as enzymatic scavengers and non-enzymatic molecules (such as glutathione).[14] Unfortunately, these protective mechanisms are not always enough to detoxify ROS and overcome the oxidative stress.[6] Highly reactive intermediates can damage cell membrane, DNA and RNA, among other targets. The effects of RF-EMF on plasma membrane structures (i.e. NADH oxidase, phosphatidylserine, ornithine decarboxylase) and voltage-gated calcium channels has been disscused.[15] Friedman *et al.* (2007) indicated that short-term exposure to RF-EMF increased the activity of plasma membrane NADPH oxidase enzyme and enhanced ROS formation.[16] In another study on the photodynamic killing of EMT6 cells, NADPH oxidase was determined as the principal source of hydrogen peroxide (H_2O_2). In addition, the inhibitory effect of extracellular catalase indicated the involvement of H_2O_2 in cell death.[17]

It seems that the magnitude of applied fields plays a more major role compared with the frequency variation in the range 835-950 GHz.[16] Moreover, it has been specifically shown that the magnetic field of low frequency EMF plays a major role in radical-pair (singlet/triplet) effects.[18]

We have previously suggested that the mobile phone frequency EMF at low intensities (E ~ 6 V/m and SAR ~ 0.025 W/kg) could affect luciferase structure and function (in-vitro). Direct interaction of applied EMF with the luciferase molecules and their hydration water has been shown to inhibit the formation of small aggregates within 30 minutes of exposure.[19]

Firefly luciferase (Fluc) has been widely used as a reporter enzyme and for the bioluminescence imaging because of its high sensitivity and quantum yield.[20-24] In this regard, there is a great

interest in studying the luciferase bioluminescence and developing prospective applications.[23-25] Recently we have designed a novel luminescent biosensor for rapid monitoring of IP3 by split-luciferase complementary assay and a whole-cell luminescent biosensor to detect the early-stage of apoptosis.[26, 27] It is also reported that, this enzyme is affected negatively following heat shock stress.[28] Therefore, it would be interesting to study the effects of EMF on living cell harbouring luciferase. The luciferase activity in the transfected 34i cells in relation with exposure (50 Hz, 1500 μT) has been studied before.[28] Luciferase activity of transfected cells has also been proven to be highly linked to oxidative stress and intracellular ROS level. Interestingly, it is also shown that firefly luciferase activity is more affected by H_2O_2 and not superoxide.[29] These studies consider luciferase as a reporter of intracellular oxidative and heat shock damage because of its sensitivity. In addition, there is no significant interference with luciferin and the luciferase enzyme in mammalian cells.[29]

Here, the time dependent (15-90 min) effects of low intensity 940 MHz EMF radiation acquired by a rectangular waveguide system on the bioluminescence and oxidative response of stable luciferase producing HEK cells were studied. The luciferase activity of cell lysate and the oxidative stress and response elements (reactive oxygen species, catalase, superoxide dismutase, reduced glutathione and malondialdehyde) were followed up in different exposure durations for exposed and non-exposed control samples.

Materials and methods

Materials

D-luciferin potassium salt, adenosine-5-triphosphate (ATP), $MgSO_4$, ethylenediaminetetraacetic acid (EDTA), penicillin/streptomycin, HEPES-buffered saline (HBS), Nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), 5, 5' dithiobisnitro benzoic acid (DTNB), thiobarbituric acid (TBA), trypsin and 2',7'-dichlorofluorescein diacetate (DCF-DA) were obtained from Sigma (St. Louis, MO, USA). Tris aminomethane and hydrogen peroxide were purchased from Merck. Dulbecco's Modified Eagle's Medium, high glucose (DMEM) from invitrogen and fetal bovine serum FBS from Gibco.

Methods

Cell culture

Human embryonic kidney cells (HEK293T cells) were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), and 1% penicillin/streptomycin in 25 cm^3 tissue culture flasks in a 5% CO_2 humidified atmosphere at 37°C. In order to have nearly uniform number of cells, the flasks were split every 2–3 days when the cells had reached ≈ 85% confluence. About 24 hours before treatment, cells were seeded into 35-mm dishes (SPL, Internal Dimension: 34.3 × 9.3 mm) at a density of 10^5 cells in 2 ml of growth medium. The growth medium was kept at 2 ml in all experiments. The cell culture and related methods used were according to previous studies.[27, 28]

Design of lentiviral vectors

To construct the pLox-cw-luciferase lentiviral vector plasmid, the PCR-amplified sequence of luciferase was derived from pGL3-Control Vector (Promega, Madison, WI, USA) using forward: 5′-CTA GGA TCC ATG GAA GAC GCC AAA AAC-3′ and reverse: 5′- GTG CTC GAG GCG ATC TTT CCG CCC TTC-3′ primers. The luciferase fragment was added between the unique *BamH1* and *Xho* Sites. Positive XL10 clones were selected by colony PCR and screened for the correct sequence by double digestion and sequencing.

Production of high titer lentiviral vectors

About 5×10^6 of human embryonic kidney cells (HEK293T) were plated in 10-cm^2 plate and grown in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO_2 to achieve a confluence of 80% after 24 h. Lentiviral transfection was performed using the calcium phosphate mediated method of 293T cells with modifications as described. Two hours before transfection, the medium was substituted with 10 ml of fresh medium preheated at 37°C. Lentivirus vector, packaging and envelope glycoprotein plasmids were mixed together in 912 µl of deionized water, 33 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA), 105 µl of 2.5 M $CaCl_2$, and 1050 µl of HEPES-buffered saline (HBS) per plate, under gentle vortexing, and pipetted into the medium in a drop – wise manner. The amount of DNA used per plate was 21 µg of lentiviral vector plasmids (pLox-cw-luciferase), 10.5 µg of packaging plasmid

(pMD2G), and 21 µg of envelope glycoprotein plasmid (psPAX2). The medium was removed 16 h after transfection and replaced with 10 ml of pre-heated medium. After that, the supernatant was harvested twice a day (morning and evening) around 72 h post-transfection and was maintained at 4°C over this period. Then supernatant was centrifuged for 10 min at 1500 rpm to remove cell debris and pass through a 0.45 µm filter. This supernatant contained virus particles and could be used for transduction.

Transduction of 293T cells

For transduction, cells were plated 24 h before transduction. Cells were about 50-60% confluent at the time of transduction. Two hours before transduction, the growth medium was replaced with warmed fresh medium. After that, 2 ml of the supernatant containing virus particles (as mentioned above) were applied drop-wise to 10-cm^2 plate and incubated at 5% CO_2, 37°C about 24 h. Following the incubation period, the medium containing the transduction mixture was changed with a fresh growth medium.

GFP expression in the control cells was visualized under a fluorescent microscope (Nikon, Japan) 24 h after transduction. The bioluminescence analysis was also performed 24 h after transduction by a luminometer (Berthold Detection Systems, Pforzheim, Germany) as reported earlier.[27, 28]

Exposure system

The details of exposure setup were discussed before.[19] Briefly, the exposure system was made up of a waveguide unit (aluminum box 600 mm in length and 170 mm in width and height), a signal generator with 0.1 Hz resolution (SMLO3, Rohde and Schwarz, Munich, Germany), and an amplifier. Two probes generated the electromagnetic wave of 940 MHz at one end of the waveguide, and the other end was matched with the same configuration (Fig. 1A). Four discs (stubs) were used for impedance matching. In this way, most of the electromagnetic wave produced by one side of the waveguide was absorbed at the other side and led out, and no wave was reflected. Having a cell monolayer inside a Petri dish results in nearly uniform exposure and a low thermal load,which can even enable the exposure of cells to SAR levels above 2 W/kg with air-cooling only.[30]

Waveguide was equipped with two fans working in opposite directions at both ends for rapid environmental atmospheric exchange (uniform temperature). The whole waveguide was placed in an incubator at 37 ± 0.3 ºC. In order to eliminate electromagnetic interference (EMI), fine metallic meshes were placed where the cooling fans joined with the waveguide system.

The spectrum analyzer (Advantest R3131, San Diego, CA, USA) was used to measure output power of the waveguide.[19] The system was simulated with computer simulation technology (CST) Microwave Studio (Version 2010, CST, Darmstadt, Germany) based on the finite integration technique (FIT).[31] In order to simulate, the 35 mm dish was filled with 2 ml DMEM, and the dielectric constant (ε') was taken to be 77.1. The input power in the experiment and simulation was 1 W. The values used for heat capacitance of the air, solution and polystyrene were 1.0, 4.18, and 1.1 J/g ºC, respectively.[19]

The magnitude of temperature rise during exposure, the electric field and the SAR (calculated using $SAR = \sigma |E|^2/2\rho$, where, σ is conductivity, ρ is density and E is RMS electric field) were obtained (Fig. 1B).

Luciferase activity measurement

In order to measure luciferase activity alterations upon the exposure, cell cultures were lysed with their paired non-exposed control after exposure durations of 15, 30, 45, 60 and 90 min. The Cell Culture Lysis Reagent (CCLR) buffer was prepared by 100 mM potassium phosphate pH 7.8, 1 mM EDTA, 7 mM 2-mercaptoethanol 1% (V/V), Tritonx-100 (1 ml/100 ml), glycerol 10% (V/V). The CCLR reagent is compatible with luciferase assay system.

The bioluminescence reaction was initiated by adding 25 µl of cell lysate to a tube containing 25 µl of Tris–HCl buffer (50.0 mM, pH 7.8) including 2.0 mM ATP, 10.0 mM MgSO4, and 1.0 mM luciferin (pH 7.8), and the light emission was recorded over 1 s with a luminometer.

Measurement of intracellular reactive oxygen species (ROS)

The DCFH-DA fluorescent probe was utilized to monitor the intracellular accumulation of ROS. DCFH-DA was dissolved and stored in the dark to prevent spontaneous hydrolysis and autoxidation. This probe can detect intracellular H_2O_2. For this purpose, the excitation and emission wavelengths of 485 nm and 530 nm were used. The suspension of the cells (1×10^6/ml) with a solution of DCFH-DA (10 µM) was incubated for 1 h at 37ºC. After rinsing the cells with

phosphate buffered saline (PBS), the fluorescence intensity was assessed by a spectrofluorimeter (Cary Eclipse50, Varian, Belrose, Australia).

The ROS content of cell suspensions was also monitored by flow cytometer (Partec space, Gottingen, Germany) after dilution with PBS. All fluorescence signals of labeled HEK293T cells were analyzed by the flow cytometer. A minimum of 1,000 cells was examined for each assay at a flow rate of < 100 cells/second. The cell population was gated using 90° and forward-angle light scatter. The excitation wavelength was 488 nm supplied by an argon laser.

Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was determined by the Kakkar method.[32] A single unit of enzyme was described as 50% inhibition of NBT reduction/mg protein. Assay mixture components were 0.1 ml of cell lysate, 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml of PMS (186 mM), 0.3 ml of NBT (300 mM) and 0.2 ml of NADH (750 mM). The reaction was started by NADH and then after 90 s incubation at 30ºC stopped by 0.1 ml of glacial acetic acid. The reaction progress was determined by the absorbance at 560 nm using UV-Vis spectrophotometer (Cary 100 Conc, Varian, Mulgrave, Victoria, Australia).

Catalase activity assay

The CAT activity was measured as previously described by Aebi.[33] Briefly, cell lysate was added to a cuvette containing 50 mM phosphate buffer (pH 7.0). The addition of freshly prepared 30 mM H_2O_2 was used to start the reaction. Measurement of the decomposition rate of H_2O_2 was performed with a spectrophotometer by monitoring the decrease in absorbance at λ= 240 nm.

Measurement of reduced glutathione

Reduced glutathione (GSH) was determined using the Ellman method (Ellman reagent, 19.8 mg of DTNB in 100 ml of 0.1% sodium nitrate).[34] The reduced glutathione reacts with DTNB (5,5'-dithiobis nitro benzoic acid) and produces a yellow coloured product with absorbance at 412 nm.

Measurement of lipid peroxidation

The lipid peroxidation was measured according to the concentration of the purple colour generated by the reaction of TBA with malondialdehyde (MDA) using a double heating

method.[35] MDA is an index of lipid peroxidation. Briefly, 0.5 ml cell lysate and 2.5 ml of tricholoroacetic acid (TCA, 10% w/v) solution was mixed up together and boiled in a water bath for 15 min. The centrifugation at 3000 rpm for 10 min was performed after cooling at room temperature. The supernatant of each sample (2 ml) was mixed with a TBA solution (0.67% w/v) in the test tube that was placed in boiling water for 15 min. The absorbance was measured at 532 nm.

Caspase 3/ 7 assay

Apoptosis was evaluated by the activity of caspase-3/7. The activity of caspase-3 and caspase-7 was detected using the Caspase-glo 3/7 detection reagent (Promega).[36] The culture medium was poured out and the attached cells were trypsinized (300 µl) for 5 minutes. In order to protect cells from trypsin, a 300 µl DEMEM culture medium was also added in this stage, and then centrifuged (5 min, 1200 rpm) to sediment the cells. The cell pellet was solved and lysed with CCLR reagent (50 µl) with 2 min pipetting. The 25 µl of cell lysate was then added to 25 µl of caspase assay mixture. The final mixture was then incubated for 15 min at 22°C and then the bioluminescence intensity was measured.

Data analysis and representation

The exposed samples in all experiments had their corresponding sham-exposed control, which were kept in the same conditions as the exposed samples except for the EMF radiation. The protein content of cell lysate was also considered in the experiments. The cell culture conditions (pH, amount of culture medium and confluence) were kept nearly similar for all experiments.

The represented figures are means of data gathered from 3 independent experiments (biological replicates; n=3) and 3 replicates (technical replicates). Comparison between control group and other groups was made by one-way analysis of variance (ANOVA) followed by the Dunnett test for all parameters. The statistical significances were achieved when $p<0.05$. Alterations in the activity of the exposed samples were illustrated considering the corresponding controls. Data are illustrated as mean ± SEM (standard error of the mean).

Results

EMF exposure simulation

The magnitude of the electric field across the 35 mm (diameter) dish was ~10 V/m (root mean square, RMS). However, in some parts, electric field intensity and SAR was higher. It is difficult to have uniform field all around the cell culture dish. Overall, the acceptable inhomogeneity for biological studies (below 30%) was satisfied.[37] The waveguide provided a 940-MHz signal at a mean SAR of ≈ 0.09 << 2 W/kg. On average, the calculated increase in temperature of the cell culture dish was 0.07 ± 0.03 °C (range, 0.02–0.1 °C), which is considered sufficiently low in observing non-thermal effects (Fig. 1B). However, two fans working in opposite directions at both ends of the waveguide homogenize the temperature.

Luciferase activity

The difference between luciferase activity of exposed and non-exposed cells treated by EMF exposure for 15, 30, 45, 60 and 90 min are depicted in Fig. 2. The change in luciferase activity (exposed - control) are illustrated in mean ± SEM. The luciferase activity of 30 and 45 min exposed cells were lower than the controls. Whereas, cells exposed to 60 min EMF showed meaningfully higher luciferase activity (0.64×10^6 $RLU.s^{-1}$). The possible effects of the waveguide system were tested when the RF signal was off (no EMF, with the fans working). There were no meaningful alterations in the luciferase activity of samples after 30 and 60 min of incubation.

Reactive Oxygen Species (ROS) and oxidative response

The results of DCF fluorescence method shows EMF could have induced intracellular ROS generation in HEK293T cells (Fig. 3). The fluorescence intensity of DCF increased about 1.86 folds until 30 min exposure, and then reduced for the longer exposure duration of 45 min. For better illustration, Fig. 3B shows the normalized peak area of the fluorescence curves. The increase in DCF fluorescence represents a statistically significant increase in ROS even from the first 15 minutes of exposure to 940 MHz EMF signal provided by the waveguide system.

For more precision, the ROS (intracellular) content of control and exposed cells were also measured by flow cytometery (Fig. 4). Panels A and B in Fig. 4 are the data from the flow cytometry of fresh HEK293T cells coloured with DCFH-DA. The flow cytometery data did not

show any change in cell size or granulosity, but there was a remarkable right shift (ROS elevation) in the fluorescence graph of 30 min exposed cells (Fig. 4D). However, similar to fluorescence curves, a decrease in ROS content in 45 min exposed HEK cells was observed (Fig. 4F), which makes the graph close to its control (Fig. 4E).

Figs. 5 and 6 demonstrate that the 45 min exposure of cells by EMF increased the activity of SOD and CAT by 1.14 and 2.64 fold respectively and then decreased after 60 min.

As Fig. 7 displays, there is a significant increase in GSH (1.22 folds) after the 45 min exposure. Therefore, the exposed cells could have activated their response to defend against oxidative stress. Meanwhile, after 60 min of continuous exposure, the GSH level was not significantly higher, which could probably be because of the cells' ability to respond to the stress was pushed to its limits. The SOD and CAT activities also show the same pattern. Fig. 8 illustrates that, MDA as a marker of lipid peroxidation decreased meaningfully for the 45 and 60 min exposed cells compared with controls.

H_2O_2 inhibits luciferase activity in μM concentrations

The in-vitro activity of luciferase was measured immediately after addition of 20-200 μM concentrations of H_2O_2. As it is shown in Fig. 9, H_2O_2 has negatively affected luciferase activity, and there is about 12% decrease in the presence of ~100 μM hydrogen peroxide. However, incubation of luciferase with H_2O_2 before activity measurement might lead to further inactivation.

Caspase 3 and 7 activities

Fig. 10 shows the EMF effect on caspase-3/7 activities. The caspase activity was increased upon longer exposure durations compared to the non-exposed control sample. Interestingly, the caspase activity peaked at 45 min and then decreased by 60 min temporarily, which was then followed by another increase after the 90 min EMF exposure.

Discussion

Apart from the well-characterized heating effects, there are no generally accepted dominant mechanisms for the interaction of RF fields with biological systems.[38] Exposure guidelines are based upon these well-established thermal effects, but the potential effects of low-level

exposures remain an area of controversy and uncertainty. In order to understand if the observed effects were due to non-thermal or thermal effect of RF exposure, the best method would be to study the effects of low intensity fields.

Studying EMF effects on the cultured cells in dishes usually provide uniformity of SAR. Fig. 1B shows the SAR is mostly uniform and below 0.2 W/kg. The amount of culture medium directly affects the SAR of a filled dish with a cellular monolayer. The obtained SAR and temperature rise are comparable to previous works by other groups.[37] However, there could be local hot spots in the sample even upon low-level EMF exposure. In addition, the increase in temperature is usually lower than the accuracy of thermal incubator used. Therefore, it might be reasonable to focus on effects which are more commonly considered as non-thermal responses.

A stable cell line (HEK293T) which continuously expresses firefly luciferase was used to monitor the effects of external 940 MHz EMF. As shown in Fig. 2, exposure of HEK293T cell lines for the shorter periods (30 and 45 min) caused a decrease in luciferase activity while longer exposure durations (60 min) led to the reactivation of luciferase compared to control cells (non-exposed). Thus, the initial loss of luciferase activity may stem from accumulation of some inhibitions or destructive compounds. Whereas upon longer exposure, EMF may activate compensatory mechanism such as induction of heat shock factors or other responsive elements. Therefore, the luciferase environment within the cells that were affected by exposure might be responsible for the observed changes in the luciferase activity.

As it is demonstrated in Figs. 3 and 4, ROS generation might be the first chemical evidence of EMF effects on HEK293T cell culture. The ROS increased and peaked 30 min after EMF exposure. Elevation of the ROS level resulted in the activation of enzymatic antioxidant defense mechanisms. The SOD converts 2 superoxide radicals ($O^{\bullet}_2^-$) into O_2 and H_2O_2, then CAT converts H_2O_2 to H_2O. GSH plays a crucial role in the antioxidant system.[4] GSH cycle is a sequence of reactions by which the degradation of H_2O_2 is coupled to the increased production of $NADP^+$ which is reduced to NADPH by the hexose monophosphate shunt (pentose phosphate pathway). Recently, it has been reported that GSH depletion stimulates the initiation phase of apoptosis in lymphoid cells.[39] Commonly, it is thought that most or all of the toxicity of $O^{\bullet}_2^-$ and H_2O_2 stems from their conversion into $OH^{\bullet}$, which is likely to be the major harmful species formed by the Haber-Weiss reaction under biological conditions.[40]

Here, SOD and CAT activity besides GSH content increased for samples exposed to 45 min EMF. All these factors activated in the cell defense system protect against oxidative injury. The flow cytometry data also indicated that the difference between ROS content was maximized during 30 min of exposure. Therefore, oxidative response is not well activated up to 30 min to compensate ROS production.

In-vitro and in-vivo studies on the concentration-response curve of dichlorofluorescein (DCFH) oxidation by H_2O_2 has been obtained previously.[41] An increase in fluorescence upon addition of H_2O_2 is caused by DCFH oxidation to DCF in a dose-dependent manner.[41, 42] Hence, the presence of ROS and H_2O_2 could explain why luciferase activity decreases in 30 and 45 min of exposure. However, after activation of the oxidative response the luciferase activity of the exposed sample reaches higher than the control ones (60 min exposure, Fig. 2).

Czupryna *et al.* have shown that direct application of H_2O_2 to HeLa cells led to a reduction in Fluc bioluminescence while H_2O_2 scavengers stabilized Fluc activity.[29] This study also reported that using Rlu8 (Renilla luciferase) is better for bioluminescent assay of apoptosis compared to Fluc because Fluc is very sensitive to ROS. However, in our study, the effects of EMF on ROS production and oxidative stress plays a major role. Peroxides formed on Trp, Tyr, and His peptides, as well as on proteins, have been shown to promote inactivation of cellular enzymes, protein cross-linking and aggregation due to the abundance of proteins within most biological systems (70% of the dry mass of most cells). Singlet oxygen ($^1O_2$) can interact with protein side-chains by physical quenching which results in energy transfer and de-excitation of the singlet state.[43]

As Fig. 6 demonstrates, the catalase activity that is responsible for the diminishing H_2O_2 was increased sharply in the exposed HEK cells in comparison with other factors in this study. Hydroxyl radical can induce strand breaks in DNA, damage proteins and cause lipid peroxidation directly or via intermediate radicals with a longer half-life. Decrease in lipid peroxidation level observed in Fig. 8 could be also considered as cell resistance to oxidative damage. This reduction is evident by a significant decrease in the MDA amount for 45 and 60 min EMF exposed cells.

After 1h of exposure to 900 MHz (1.35 - 10.8 W/kg) a dose-response increase in caspase 3 activity has been observed.[44] Another study used a 900 MHz signal at lower SAR of 0.25 W/kg showed no difference in apoptosis between exposed and unexposed cells.[45] The authors conclude that induction of apoptosis could be independent of the changes in temperature while, localized

thermal effects of the RF field could not be ruled out. Typically, there is a known relation between increase in caspase activity and decrease in the cell viability. However, the elevation of caspase 3/7 could be related to cell development [46] and differentiation.[47]

Apoptosis was induced by doxorubicin in HEK293T cells to estimate the caspase 3/7 activity in apoptotic conditions, the caspase 3/7 activity was significantly higher after 1h of treatment with doxorubicin 10 μM (compared to EMF exposure). Therefore, the observed increase in caspase 3/7 activity could not be attributed to remarkable induction of apoptosis by exposure especially when there was no noticeable change in cell viability of exposed and control cells. However, the possibility to induce apoptosis cannot be ignored for long-term treatments. Tusi *et al.* showed that H_2O_2 (150 μM, 24 h) can induce caspase-independent and caspase-dependent (ER and mitochondrial-mediated) apoptotic cell death (differentiated PC12 cells), which was largely inhibited by alginate oligosaccharide pretreatment.[48] Another study also showed that H_2O_2 (250 μM, 4 h) severely induced apoptosis of the primary Leydig cells in vitro.[49]

Heat shock responses are very sensitive to an increase in temperature.[12] There is a controversy of results in different studies for the induction and expression of heat shock proteins. Therefore, it is necessary to use a bioluminescent protein like luciferase to understand intracellular effects (damage) caused by electromagnetic fields. That is why, in an attempt to question the toxic effect of heat shock and related stress, the activity of luciferases as a reporter enzyme (Plasmid pRSV/L carries a cDNA coding for *P.pyralis* luciferase under control of the Rous sarcoma virus LTR promoter) has been studied before.[50]

Recently, it has been shown that RF could induce adaptive response (AR) in mammalian cells exposed to ionizing radiation.[51-53] Human HL-60 cells were pre-exposed to 900 MHz RF at 12 $\mu W/cm^2$ (0.12 W/m^2) power density for 1 h/day for 3 days and then treated with doxorubicin. Exposed cells showed a decrease in apoptosis and intra-cellular Ca^{2+} and an increase in proliferation, mitochondrial membrane potential and Ca^{2+}–Mg^{2+}-ATPase activity as compared with the cells treated with doxorubicin (challenge dose, CD) alone.[54] Sannino *et al.*[55, 56] treated the human blood lymphocytes with phytohemagglutinin for 24 h and then exposed them to 900 MHz for 20 h (adaptive dose, AD), the incidence of micronuclei was significantly lower in lymphocytes exposed to AD + CD compared to those exposed to CD alone. Mortazavi *et al.* represented the induction of adaptive response in animal studies.[57]

Enhancement in cell survival (40-50%) and diminished apoptotic response in H9c2 cells by magnetic field preconditioning (120 μT, 4-8 h) was also indicated recently. However, levels of Hsp's 25, 32 and 72 showed little-to-no elevation (compared with ischemia and reperfusion as a challenging element which induce apoptosis).[58]

The mechanism by which RF or magnetic fields are able to induce AR needs much more investigations. Perhaps, RF exposure given as AD leads to a condition that does not induce significant damage, but offers a protection to the subsequent damage by other stressful conditions. Therefore, we can suggest that the adaptive response could be related to the induction of oxidative responses in RF exposed cells. The exposure with EMF induces oxidative stress in cell, if the absorbed dose is lower than the threshold of cell oxidative response capacity, cells could be temporary more adapted to the lethal dose.

Experiments focusing on non-thermal effects should be performed without significant increase in temperature. The ICNRP guidelines have been established to guarantee that the whole body temperature increase will be below about 1°C and claim that in adult healthy people this rise does not disturb the thermal equilibrium. In fact, a 2 W/kg localized exposure may cause physiologically meaningful heating in an organism (therefore the whole body SAR is set at 0.08 W/kg) for public exposure, while values for workers are 0.4 and 10 W/kg. These values do not apply to in vitro exposure, but only to exposure of parts or whole of the human body.

For in-vitro conditions, where a small amount of sample (2 ml in a Petri dish) and enhanced atmospheric exchange (cooling fans) is used, the temperature elevation upon RF exposure in the cell monolayer would be much smaller than in the samples used for guidelines. In addition, the convection currents in the cell culture medium would homogenize temperature. Hence, low SAR exposure of cell monolayers inside Petri dishes apparently does not lead to a significant temperature increase.[30] Therefore, the observed alterations in luciferase activity, ROS content and the oxidative stress elements for exposed and control cells are more probably due to non-thermal effects of 940 MHz EMF exposure.

It should be noted that a little unavoidable increase in temperature could not be completely omitted from the observed effects. Therefore, the stress-related cascades that are known to be triggered by heat or other related stresses could be activated in the time course of experiments especially for studies with long EMF treatments.[16]

In order to find out possible advantages and the interaction mechanisms of EMF, the minimum SAR (or intensity) that could have biological effect (MSBE) would be much more valuable in comparison to studying high intensity fields. Such studies can possibly shed light on thresholds of non-ionizing radiation effects, and cell capabilities (e.g., oxidative response). In addition, it is more likely to reduce the complexity of the EMF interaction targets in cell cultures by lowering the exposure power, which at least reduces the overall rise in temperature.

Conclusion

Based on the results presented, it could be concluded that a stable cell line that persistently expresses firefly luciferase can act as a suitable host to monitor stress responsive elements upon exposure to an external EMF field. Luciferase is both sensitive to ROS (H_2O_2) and other intracellular factors which may favour protein misfolding during the stress. The effect of different exposure durations on the oxidative stress factors and luciferase activity indicated that there is an order in the effects caused by applying 940 MHZ EMF and the cell responses. It seems that EMF exposure initially caused a burst in the ROS content of cells, and consequently decreased the luciferase activity. Oxidative stress response factors (CAT, SOD and GSH levels) are elevated to reduce ROS content. This compensation in HEK293T cells was observed after 45 min of continuous exposure to low intensity 940 MHz EMF. Concordantly, an index of lipid peroxidation (MDA) was also reduced after activation of oxidative response. Thus, if the exposure dose is lower than the cell oxidative response capacity, cells could be temporarily adapted to the lethal dose or even other stress conditions. This observation might clarify the adaptive response mechanism in combined EMF and ionizing radiation studies.

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Legends:

Fig.1A) Model of waveguide with 1) excitation probes, 2) 35 mm Petri dish and 3) four impedance matching discs (stubs). B) Simulated SAR (W/Kg) distribution in 35 mm cell culture dish filled with 2 ml of DMEM.

Fig.2 Time dependent effect of EMF exposure on bioluminescence of luciferase producing HEK293T cells. Each value represents the mean ± SEM (n=3). [***P < 0.001, **P < 0.01, *P < 0.05 different from the control group].

Fig.3 A) Time dependent effect of EMF exposure on fluorescence intensity of DCFH-DA as a probe of intracellular ROS, excitation at 485 nm. The curves are mean of independent experiments and corrected for protein concentrations of each sample. B) The normalized peak areas calculated from fluorescence curves. Each value represents the mean ± SEM. [***P < 0.001, **P < 0.01 different from the control group].

Fig.4 Flow cytometer graphs of control and exposed HEK293T cells with intracellular DCFH-DA fluorescent dye after 1 h incubation at 37 ºC. Panels A and B are relate to fresh cells, fluorescent particles and gating region are demonstrated. Panel C and E show control cells accompanied by their 30 and 45 min exposed ones in panel D and F, respectively. The flow cytometer statistical data is presented.

Fig.5 Time dependent effect of EMF exposure on superoxide dismutase (SOD) activity of HEK293T cells. Each value represents the mean ± SEM (n = 3). [**P < 0.01, *P < 0.05 different from the control group].

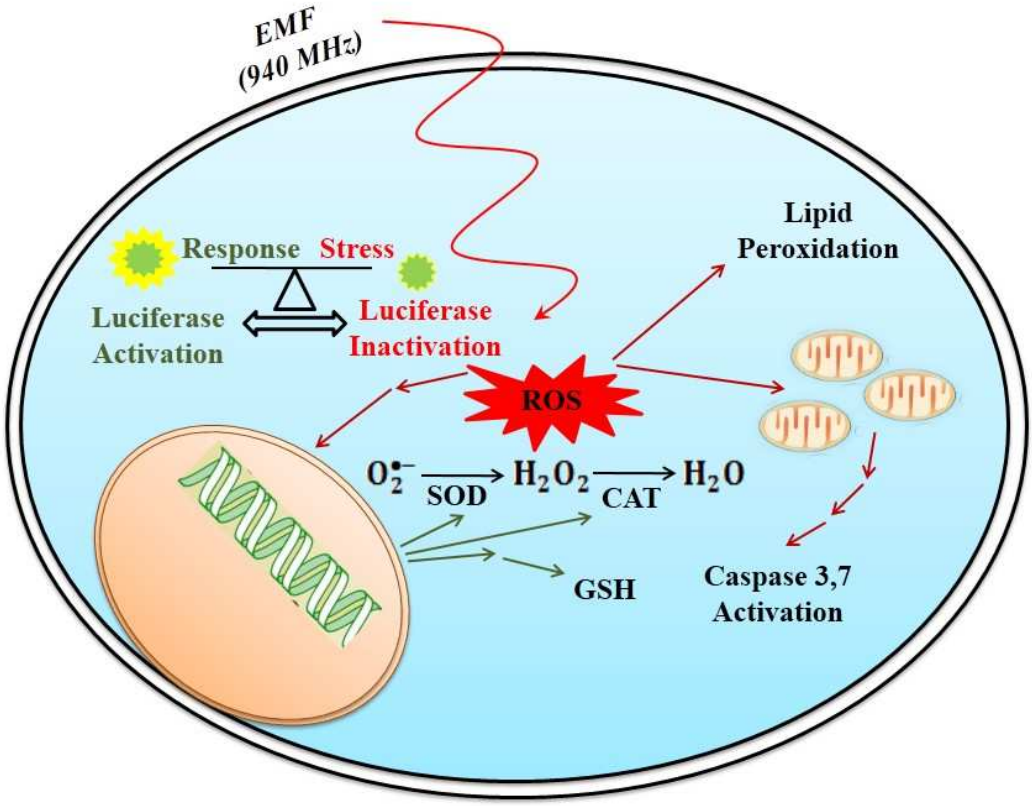
Fig.6 Time dependent effect of EMF exposure on catalase (CAT) activity of HEK293T cells. Each value represents the mean ± SEM (n = 3). [***P < 0.001, **P < 0.01 different from the control group].

Fig.7 Time dependent effect of EMF exposure on reduced glutathione (GSH) level of HEK293T cells. Each value represents the mean ± SEM (n = 3). [*P < 0.05 different from the control group].

Fig.8 Time dependent effect of EMF exposure on malondialdehyde (MDA) level of HEK293T cells. Each value represents the mean ± SEM (n = 3). [**P < 0.01, *P < 0.05 different from the control group].

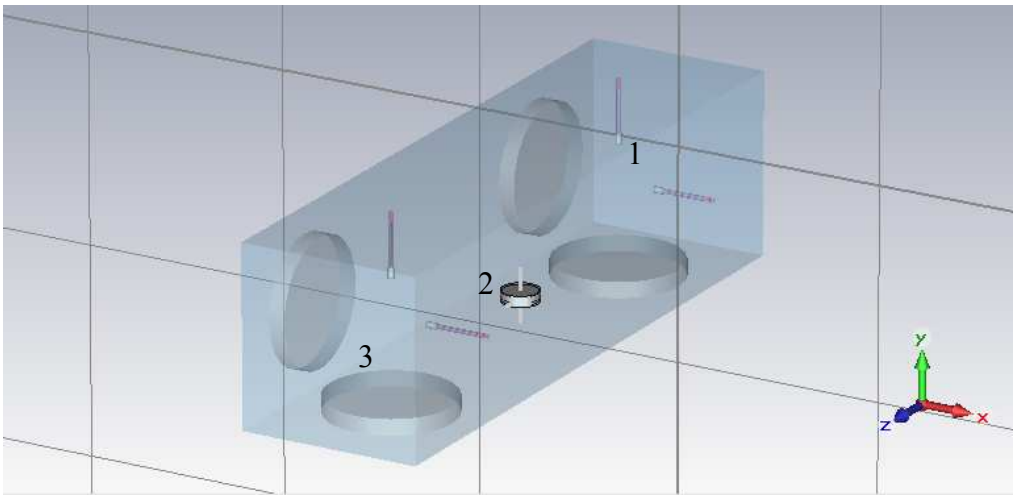
Fig9. Effect of H_2O_2 on luciferase activity. Each value represents the mean ± SEM (n=3).

Fig.10 Time dependent effect of EMF exposure on caspase 3/7 activity of HEK293T cells. Each value represents the mean ± SEM (n=3). [***P < 0.001, *P < 0.05 different from the control group].



Oxidative stress and response are among EMF mechanisms of action, the absorbed dose and ability of cells to responded might be summarized in intracellular luciferase activity.

Fig. 1



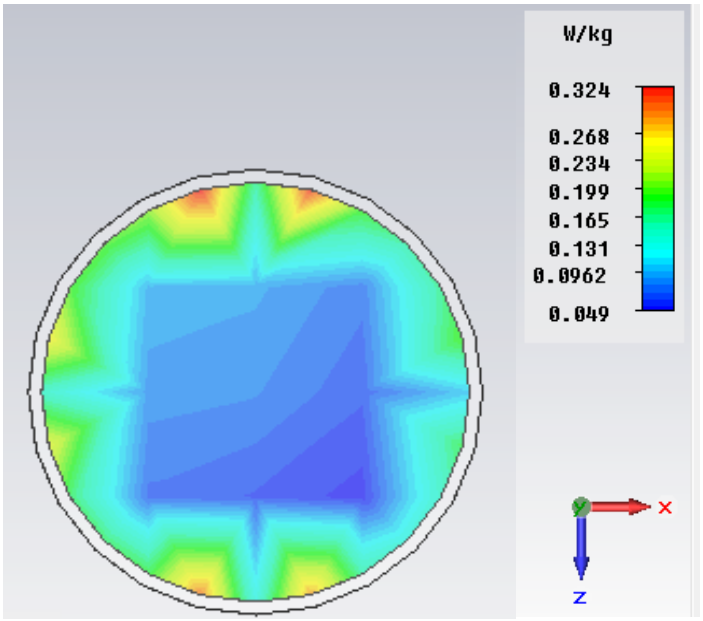


Fig. 2

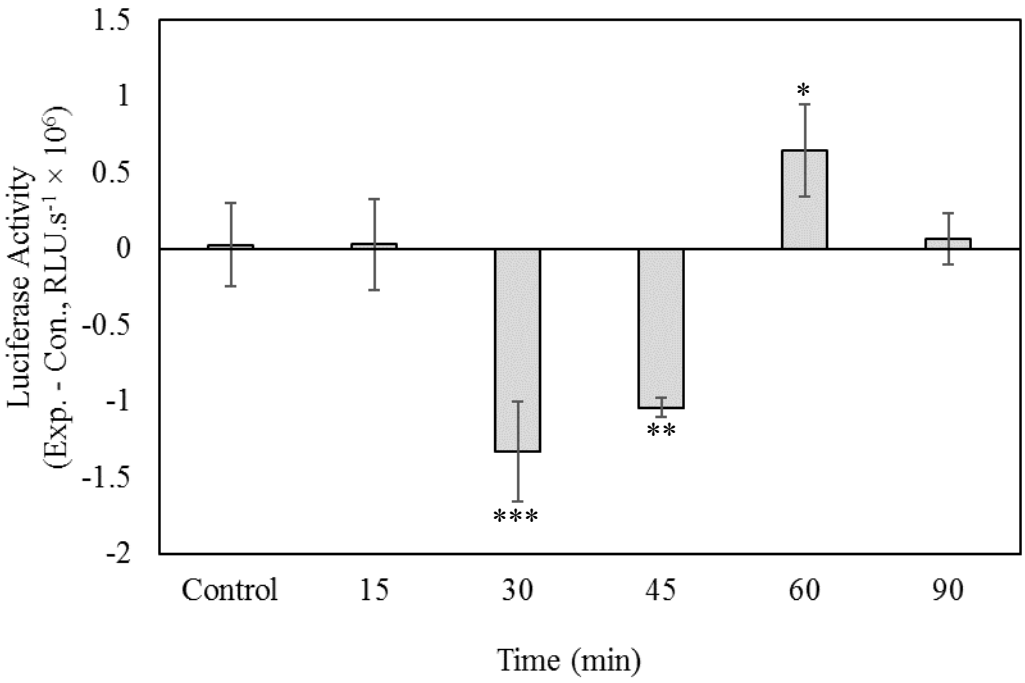
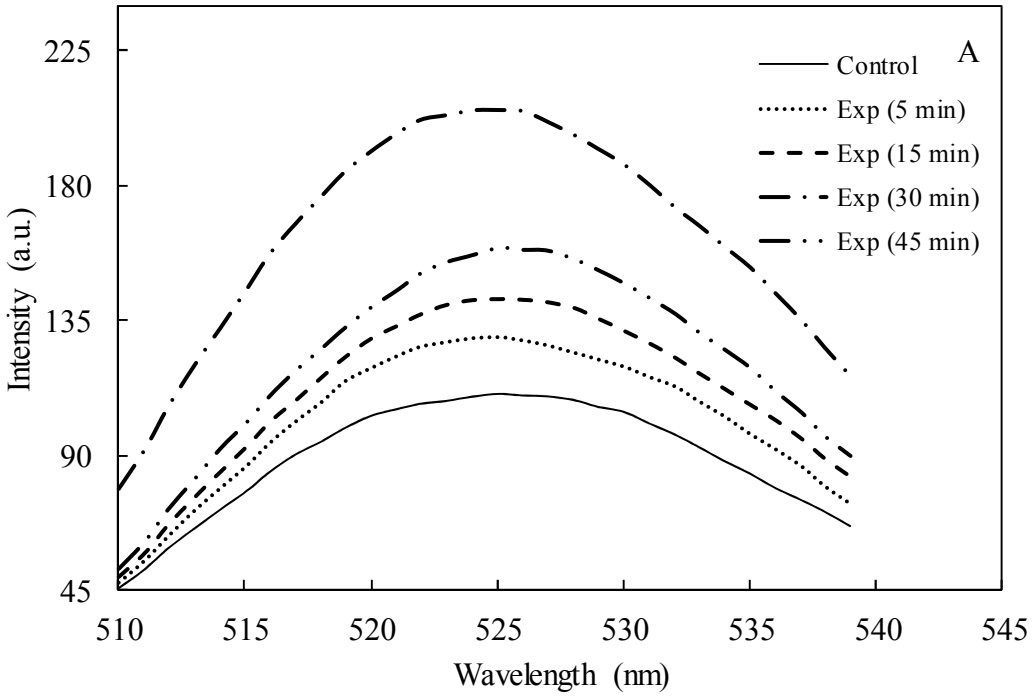


Fig. 3



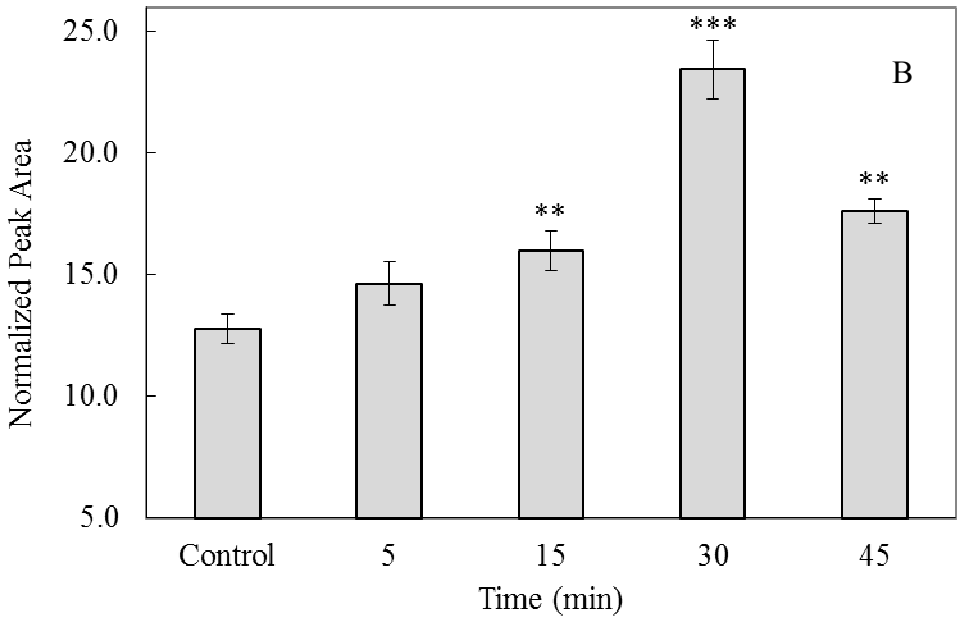
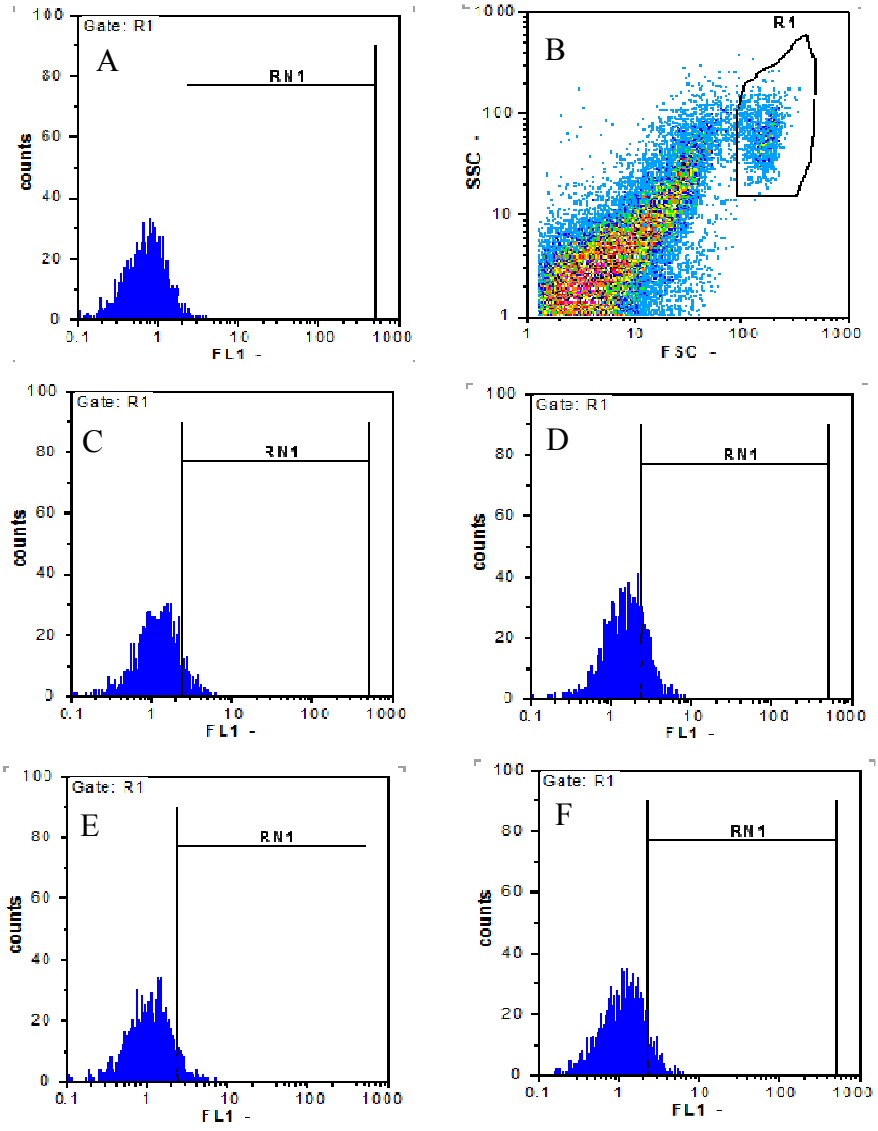


Fig. 4



Time (min)	R1 Counts		RN1 counts		Percent in RN1 (%) ± 5	
	Con.	Exp.	Con.	Exp.	**Con.**	**Exp.**
5.0	1423	5776	44	183	**3.09**	**3.17**
30.0	2095	2651	200	516	**9.55**	**19.46**
45.0	2123	2245	154	206	**7.25**	**9.18**

Fig. 5

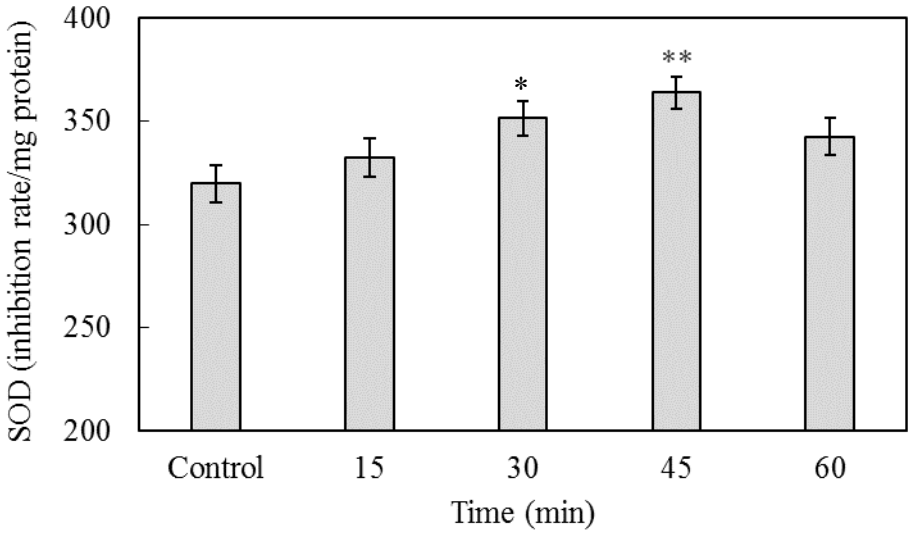


Fig. 6

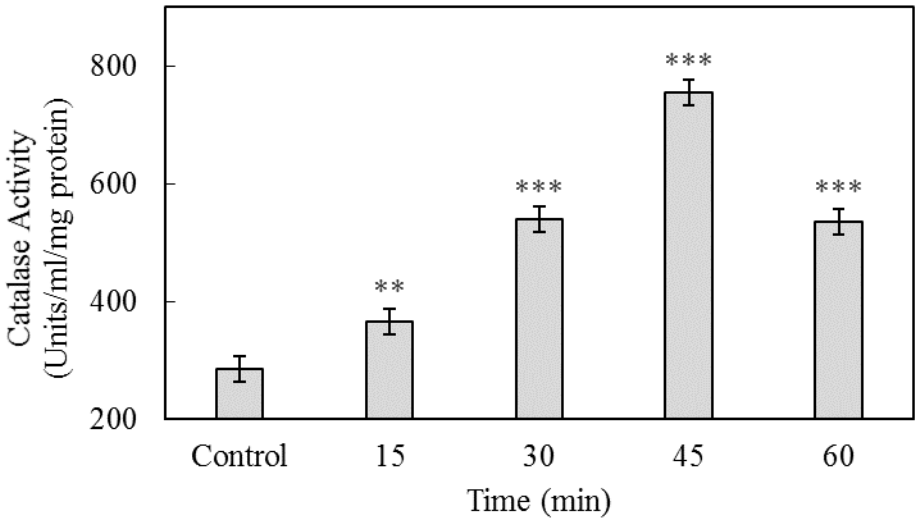


Fig. 7

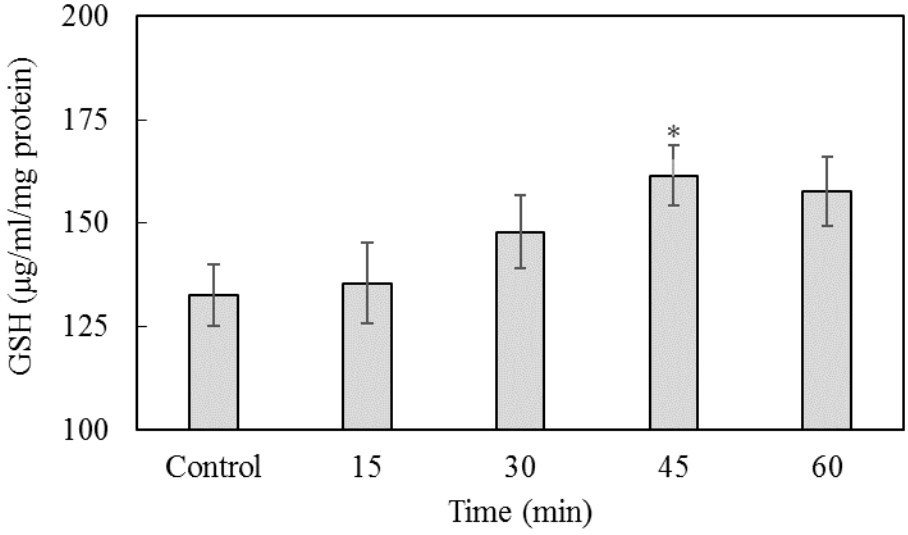


Fig. 8

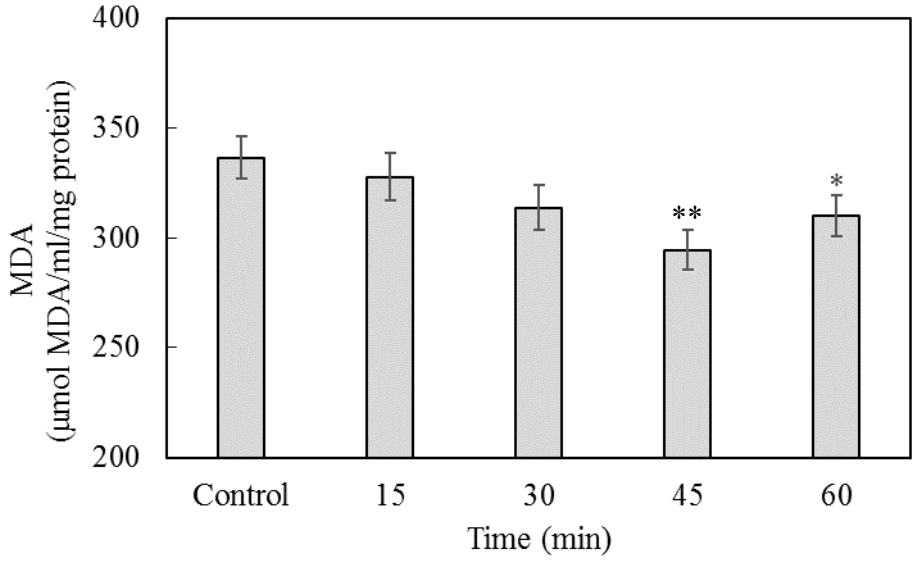


Fig. 9

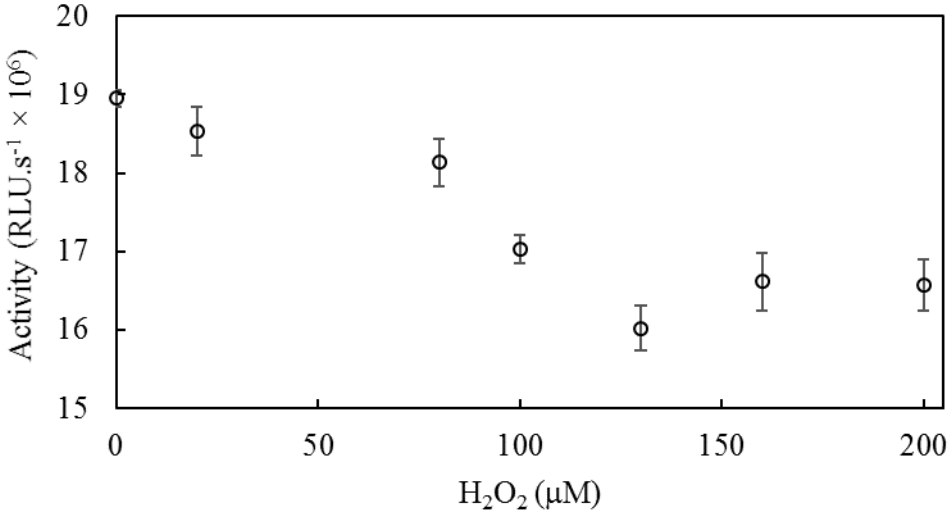


Fig. 10