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Cytoprotective effects of low-frequency pulsed electromagnetic field against oxidative stress in glioblastoma cells

Çiğdem Gökçek-Saraç[1], Tuğçe Şimşek[1] and Serdar Karakurt[2]

[1] *Department of Biomedical Engineering, Faculty of Engineering, Akdeniz University, Antalya, Turkey*
[2] *Department of Biochemistry, Faculty of Science, Selcuk University, Antalya, Turkey*

Abstract. The low-frequency pulsed electromagnetic field (PEMF) may have possible cytoprotective effects against the destructive effects of oxidative stress. The goal was to investigate if short-term low-frequency PEMF has cytoprotective effects in glioblastoma cell line following high-dose hydrogen peroxide (H_2O_2) treatment. U87-MG cells were divided into four groups: Sham-control group; PEMF group (cells exposed to PEMF); H_2O_2 group (cells treated with H_2O_2 at time intervals 30 min and 48 h, respectively); H_2O_2+PEMF group (cells exposed to PEMF after H_2O_2 treatment at time intervals 30 min and 48 h, respectively). The cell viability, levels of reactive oxygen species, glutathione peroxidase activity, and the amount of glutathione were measured. The cytoprotective effect of PEMF against deleterious effects of oxidative stress triggered by different time interval of H_2O_2 treatment might be mediated by the increase in the cell viability, the elevation in the antioxidant enzyme activity/amount, and the decrease in the reactive oxygen species level. In addition, the cytoprotective effect of PEMF varies depending on different time intervals of H_2O_2 treatment. In the light of these findings, further *in vivo* and/or *in vitro* studies on neurophysiological effects of PEMFs and their underlying molecular mechanisms are needed to elucidate neurotoxic or neuroprotective role against antioxidant defense mechanisms.

Key words: Pulsed electromagnetic field — U87-MG cells — Oxidative stress — Reactive oxygen species — Antioxidants

Introduction

Nowadays, investigating cytotoxic and/or cytoprotective effects of extremely low-frequency electromagnetic fields (ELF-EMF) on living tissues depending mainly on dose and timing continues to attract the attention of researchers. Some researchers demonstrate the beneficial effects of low-frequency electromagnetic field exposure, such as treating diverse types of pain (Ehnert et al. 2019; Gessi et al. 2019; Kohli et al. 2020), and its possible inhibitory effect on the proliferation of the glioblastoma cancer cell line (U251) (Makinistian et al. 2019) as well as others showing its harmful effects, including the risk of developing brain cancers and/or neurodegenerative diseases (Gessi et al. 2019; Kim et al. 2019). Particularly, cellular responses vary depending on the cell type, tissue or organism examined (Akdağ et al. 2013). Pulsed electromagnetic fields (PEMF) of extremely low-frequency are non-ionizing and use series of magnetic pulses of electrical energy into exposed biological tissue to accelerate tissue repair without induced thermal effects (Ehnert et al. 2019). It has been used as an alternative biotherapy for various medical problems, including post-operative pain relief with its anti-inflammatory effects in addition to reducing tumor growth, cell proliferation, cytotoxicity, and apoptosis in cancer (Vincenzi et al. 2017). Moreover, it has been shown that PEMF exposure depending on dose, timing, and exposure conditions suppressed the production of reactive oxygen species (ROS) whose overproduction may affect cellular elements thoroughly by damaging antioxidant

Correspondence to: Çiğdem Gökçek-Saraç, Faculty of Engineering, Department of Biomedical Engineering, Akdeniz University, 07058 Antalya, Turkey
E-mail: gokcekcigdem@gmail.com

mechanisms (Osera et al. 2015; Vincenzi et al. 2017). For example, short-term exposure to ELF-EMF has been reported to activate systems that control oxidative balance in rat brains (Martinez-Samano et al. 2012), prevent apoptotic death without any change in ROS production in lymphocyte cells (Palumbo et al. 2006), and activate redox-based adaptive responses in neuroblastoma cells (Sulpizio et al. 2011; Osera et al. 2015; Falone et al. 2016), whereas long-term exposure has been reported to weaken antioxidant defenses of aged rats' brain through ROS overproduction (Falone et al. 2008) and induce apoptosis in human umbilical cord blood lymphocytes without any effect on ROS levels in the specific range of field intensities, suggesting "amplitude window effect" (Zastko et al. 2020). Nevertheless, the mitochondrial Mn-dependent superoxide dismutase's (MnSOD) positive redox enzymatic response has been found in young rats' brains after chronic (10 days) exposure to 50 Hz magnetic field (Falone et al. 2008). In addition, short and repeated pre-exposure of neuroblastoma cells (SH-SY5Y, SK-N-BE(2)) to 75 ± 2 Hz frequency, 2 ± 0.2 mT intensity of PEMF resulted in a decrease in ROS production and an increase in the Mn-SOD-based antioxidant production following a pro-oxidant challenge (Osera et al. 2015; Falone et al. 2016). It is worthy to note that the drug-resistant SK-N-BE(2) neuroblastoma cell line showed intense PEMF-induced MnSOD-dependent antioxidant activity as compared to the drug-sensitive SH-SY5Y cancer-derived cell line (Osera et al. 2015; Falone et al. 2016). These findings indicate whether low-frequency PEMF exposure may have cytotoxic and/or cytoprotective effects which depend on the timing, dose, and study design. Still, more studies are needed to investigate the potential effects of low-frequency PEMF on biological functions.

In the central nervous system (CNS), there are two main types of antioxidant responses, one of them includes enzymatic and the other one includes non-enzymatic responses (Chen et al. 2020). The most efficient intracellular enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), while in the non-enzymatic antioxidant responses, glutathione (GSH), which is oxidized by GPx to glutathione disulfide, plays crucial roles (Ighodaro and Akinloye 2017; Zhang et al. 2019). Furthermore, neuronal cells are particularly susceptible to oxidative stress due to their excessive oxygen demand and inadequate defense mechanisms against free radicals (Salazar-Ramiro et al. 2016). Astrocytes, type of glial cells, have crucial roles in regulating oxidative stress in CNS as well as providing physiological support to neurons (Salazar-Ramiro et al. 2016). One of the most common types of malignant astrocytic brain tumors in adults is glioblastoma multiforme (GBM) (Ahmadi-Zeidabadi et al. 2019).

Previous *in vitro* experimental studies have shown that exposure to different doses of PEMF in different time durations may have different biological effects on redox status (Osera et al. 2015; Falone et al. 2016). In such studies, pre-exposed effects of PEMF on oxidative stress parameters have been investigated using different molecular approaches (Osera et al. 2015; Falone et al. 2016; Ehnert et al. 2017; Vincenzi et al. 2017). However, to date, there have been no reports on the effects of certain doses of PEMF exposure within certain periods in human glioblastoma astrocyte-like cells where oxidative stress was primarily induced by high dose hydrogen peroxide (H_2O_2) treatment at different time intervals. Therefore, herein different from other studies we hence want to explore the possible direct effects of short-term (15 min) exposure to low-frequency (75 Hz; 1 ± 0.2 mT intensity) PEMF on oxidative stress induced glioblastoma U87-MG cells by measuring cell viability, ROS levels, GPx activity, and GSH amount.

Materials and Methods

PEMF exposure system

PEMF system (Pasco; UI-5000) with its software (Pasco Capstone; UI-5400) was purchased from Edutek Company, Ankara, Turkey. The Helmholtz coils, consisting of two 500 turns pairs, 0.64 mm diameter copper wire with a total diameter of 20 cm, were placed parallel to each other to generate a homogeneous magnetic field environment for the cells to be exposed. Each coil was then connected in series and powered by a Pasco 850 Universal Interface pulse generator (Pasco; UI-5000). The software was used to determine the 75 Hz frequency pulse signal with a pulse duration of 1.3 ms. The amplitude was set as 15 V. The intensity peak of 1 ± 0.2 mT was measured between two coils using the Pasport 2-Axis Magnetic Field Sensor with an accuracy of 0.2% to ensure homogeneity in a coil system where cultures were placed. The shape and impulse length of the induced electric field were kept constant. The coil system was then placed into the cell incubator and the ambient temperature was set as 37°C to eliminate the temperature effect through cells. In addition, to ensure that hyperthermia is not responsible for the PEMF effects, the local temperature in the culture medium was measured using a temperature sensor during experiments. The set-up was illustrated in Figure 1. The applied set-up was compatible with the set-up from literature (Karaman et al. 2018).

Cell cultures and treatments

U87-MG cell line (ATCC-American Type Culture Collection Manassas, VA, USA) of human glioblastoma cells was cultured in 75 cm^2 flasks (Corning, New York, USA) in Eagle's Minimum Essential Medium (EMEM; Biological Industries, Cromwell, USA). The cultures were then supplemented with

10% (v/v) inactivated fetal bovine serum (FBS; Biological Industries, Cromwell, USA), 1% (v/v) penicillin-streptomycin (Pen-Strep; Biological Industries, Cromwell, USA), and 2 mM L-glutamine (Biological Industries, Cromwell, USA). Cells were incubated in a 95% humidified atmosphere of 5% CO_2 at 37°C in an incubator (Binder, USA) for 48 h.

Cells were then transferred into 96-well plates, and experimental groups were settled as: Sham-control group; PEMF group: cells exposed to 75 Hz, 1 ± 0.2 mT PEMF for 15 min; H_2O_2 group: cells treated with 0.1 mM H_2O_2 at two different time intervals (30 min, and 48 h, respectively); H_2O_2+PEMF group: cells post-exposed to 75 Hz, 1 ± 0.2 mT PEMF for 15 min following 0.1 mM H_2O_2 treatment at two different time intervals (30 min, and 48 h, respectively). Cell cultures were placed at the coil system. The sham-control groups of cells were placed at the coil system, incubated under the same experimental conditions but without PEMF exposure.

Cell viability

Cells were trypsinized and counted by Bio-Rad TC20 automatic cell counter (Bio-Rad, California, USA) using 1:1 dilution in 0.04% Trypan Blue (Sigma-Aldrich, Missouri, USA), and then seeded (1×10^4/well) into 96-well plates as three replicates and incubated at 37°C for 24 h. Alamar Blue reagent (Invitrogen, Thermo Fischer Scientific, Waltham, MA, USA) was used as an indicator to determine the cell viability and cytotoxicity of compounds using a multi-well scanning spectrophotometer (Multiskan Go; Thermo Scientific Co., Waltham, MA, USA) with excitation and emission at 570 nm and 610 nm, respectively. Cells were then monitored by automated cell counter. Results were expressed as a percentage cell viability relative to the sham-control group.

Determination of ROS levels

Cells in each group were seeded into a 96-well plate (1×10^4 cells/well) and were rinsed with 10 mM PBS. The levels of ROS were measured using the fluorescent probe 2',7'-dichlorodihydrofluorescence diacetate (DCFH-DA; Sigma Aldrich, Missouri, USA) which is based on the principle of oxidation of DCFH to fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. In this way, the reactive fluorescence unit (R.F.U.) in cells can be determined. A mixture of 10 µM DCFH-DA was prepared (0.19 mg of DCFH-DA dissolved in 40 ml of dimethyl sulfoxide (DMSO)), and then added to Petri dishes containing cell groups. The Petri dishes were then incubated for 30 min at 37°C in 5% CO_2. Subsequently, cells were again rinsed with PBS and then seeded into a dark 96-well plate (1.25×10^3 cells/well). The fluorescent intensity was measured by Synergy H1 Microplate Reader (BioTek, Vermont, USA) with excitation and emission at 504 nm and 529 nm, respectively.

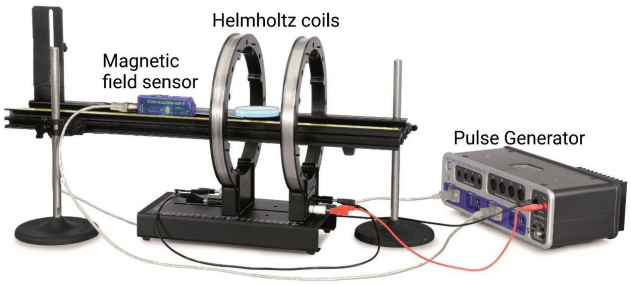

Figure 1. Representative illustration of PEMF exposure set-up with a cell culture dish between Helmholtz coils.

Measurement of GPx activity

GPx activity in cells was measured *via* ABCAM Glutathione Peroxidase Assay Kit (ab102530; Abcam; Cambridge, UK). First, cells were washed with 10 mM cold PBS. After adding 200 µl cold glutathione peroxidase assay buffer to cells, cells were centrifuged at 10,000 × *g* for 15 min at 4°C. Then, collected supernatants were transferred as duplicated to a 96-well plate. Incubation was conducted for 15 min at 37°C in 5% CO_2 after addition of 40 µl fresh colorimetric reaction mix to each well. Next, a solution of cumene hydroperoxide (10 µl) was added to each well, and plates were spectrophotometrically (Thermo Fisher Scientific, Waltham, USA) read at 340 nm. Dark incubation at 25°C for 5 min was then conducted. Subsequently, the plates were read at 340 nm, and finally, the GPx activity was expressed as mU/ml using the manufacturer's introduction.

Determination of GSH amount by high performance liquid chromatography

The amount of GSH in cells was determined using Shimadzu, LC-2050 HPLC system (Kyoto, Japan). The samples and standards were separated by an InertSustain C18 analytical column (150×4.6 mm, 5 µm; Kyoto, Japan) with a mobile phase including 25 mM K_2HPO_4 (pH 5.5) and methanol (70:30 v/v). Cells were washed with 10 mM PBS and then centrifuged at 400 × *g* for 5 min. 300 µl chloroform was added to the discarded supernatants and then supernatants were centrifuged at 10,000 × *g* for 15 min. After addition of 100 µl of trichloroacetic acid (TCA) to supernatants and the centrifugation step of mixture at 16,300 × *g* for 10 min, 500 µl Tris HCL (0.5 mM, pH 0.89) and 0.5 M K_2HPO_4 (pH 8, including 10 mM DTNB) were added to the newly obtained supernatants and supernatants were incubated on ice for 5 min. Then, 100 µl of 7 M H_3PO_4 was added to the supernatant and centrifuging was carried out at 3000 × *g* for 10 min. The supernatants were then incubated on ice till GSH quantification. The quantitative analysis of GSH was conducted using a calibration curve constructed by 5 different

concentrations of stock GSH standard solution. Supernatants and standard solutions were first filtered through 2 μm membrane followed by the addition of 0.5 ml Ellman's reagent (0.5 mM) and then GSH amount was measured according to the peak value at 4.5 min of the chromatogram obtained at 330 nm by using HPLC (Shimadzu, LC-2050, Kyoto, Japan).

Statistics

All experiments were repeated at least in triplicate. Data analysis was performed using a One-Way Analysis of Variance (ANOVA) with a *post-hoc* Tukey's Honestly Significant Difference (HSD) test, when appropriate. Data were represented as means ± SD. SPSS software (version 23.0) was used for all statistical analyses. * $p < 0.05$ was considered to be a significant difference.

Results

PEMF exposure conditions of cells

Biot-Savart law that briefly defines the magnetic field produced by a constant electric current was used to determine the magnetic field, which can be calculated by

$$d\vec{B} = \frac{\mu_0}{4\pi}\frac{Id\vec{l}\,x\,\hat{r}}{r^2} \quad (1)$$

where $d\vec{B}$ is the magnetic field contribution, $\vec{dl}$ refers to the small segments of the wire, I is the current, r is the distance from the reference point to the magnetic field, $\hat{r}$ refers to the corresponding unit vector, and μ_0 refers to the permeability of free space constant ($\mu_0 = 4\pi\times10^{-7}$ T·m/A), respectively.

In Pasco Capstone software, the waveform, the frequency, the amplitude, and the pulse duration were selected as a square, 75 Hz, 15 V, and 1.3 ms, respectively. The current flow passed through Helmholtz coils was set as 1.5 A. The magnetic field was measured by Pasport 2-Axis Magnetic Field Sensor, and then the magnetic field value was monitored as 1 ± 0.2 mT according to the software. Hence, we confirmed that PEMF and H_2O_2+PEMF group of cells were exposed to 1 ± 0.2 mT intensity, 75 Hz frequency of PEMF with a duration of 15 min.

Effect of post-exposed PEMF on cell viability

A time-dependent decrease in cell viability was observed in H_2O_2 group and H_2O_2+PEMF group as compared to the sham-control group and PEMF group ($p \leq 0.001$, respectively) (Fig. 2). In H_2O_2 group, the H_2O_2 treatment generated reduction in cell viability and this reduction was most produced at 48 h H_2O_2 treatment ($p \leq 0.001$). Interestingly, the reduction in cell viability was not as much as in H_2O_2+PEMF group. Accordingly, in H_2O_2+PEMF group, reduction in cell viability was again most produced at 48 h ($p \leq 0.001$). Besides, there is not any significant reduction in cell viability when cells just exposed to PEMF without H_2O_2 treatment (PEMF group) which indicated cytoprotective effect of PEMF exposure. According to overall results, we hypothesized that low-frequency PEMF exposure after H_2O_2 treatment has a possible cytoprotective effect on cell viability against oxidative stress and this effect may vary depending on different time intervals of H_2O_2 treatment.

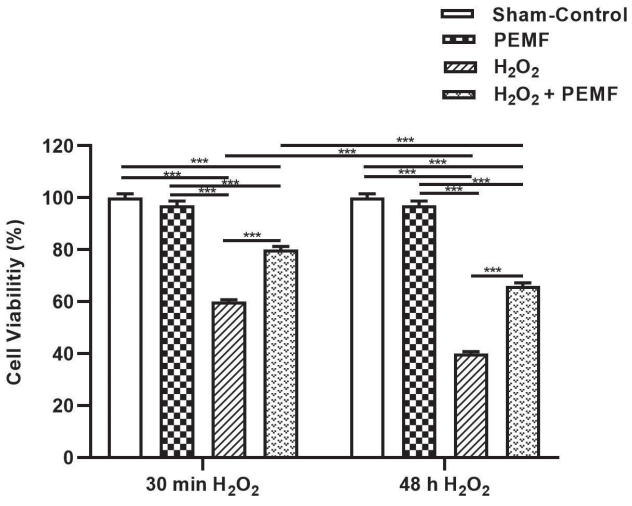

Figure 2. The percentage of cell viability in experimental groups measured by Alamar Blue assay in U87-MG human glioblastoma cell line. Results are representative of three independently performed experiments. *** $p \leq 0.001$. PEMF, pulsed electromagnetic field; H_2O_2, hydrogen peroxide.

Effect of post-exposed PEMF on intracellular ROS levels

Spectrophotometrically determined intracellular ROS levels in all groups were represented in Figure 3. Overall results have shown that ROS levels in cells (H_2O_2 group and H_2O_2+PEMF group) were significantly higher as compared to sham-control group ($p \leq 0.001$, $p \leq 0.001$, respectively). It has been observed that levels of ROS increase with the induction of oxidative stress by H_2O_2 treatment with different time intervals. Especially, treatment of cells with H_2O_2 for 48 h significantly increased ROS level as compared to 30 min treatment ($p \leq 0.01$). The ROS level in cells exposed to low-frequency PEMF after H_2O_2 treatment was significantly lower than cells treated with only H_2O_2 ($p \leq 0.01$). Since treating cells with H_2O_2 for a long time, such as 48 h, may be likely to cause an increase in ROS levels, the ROS level observed at 48 h after H_2O_2 treatment is significantly higher than 30 min in cells exposed to PEMF after H_2O_2 treatment ($p \leq 0.01$). This shows that the cytoprotective effect

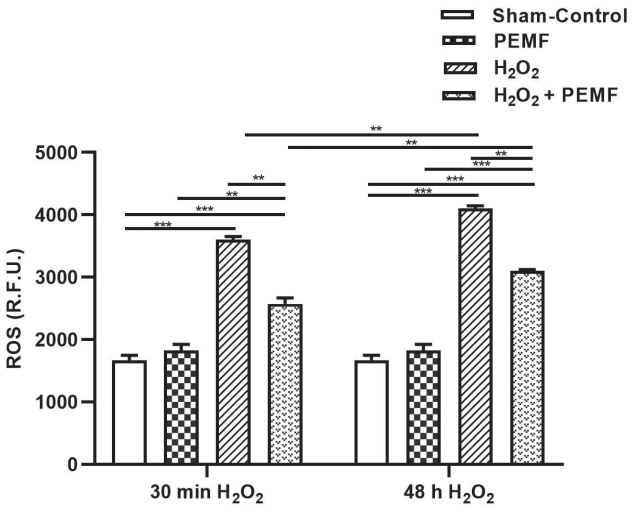

Figure 3. Comparison of the reactive oxygen species (ROS) levels in U87-MG cell groups. Data were expressed as the mean ± SD, and were analyzed by One-Way ANOVA with Tukey's HSD *post-hoc* test. ** $p < 0.01$; *** $p \leq 0.001$. All experiments were repeated at least in triplicate. PEMF, pulsed electromagnetic field; H_2O_2, hydrogen peroxide.

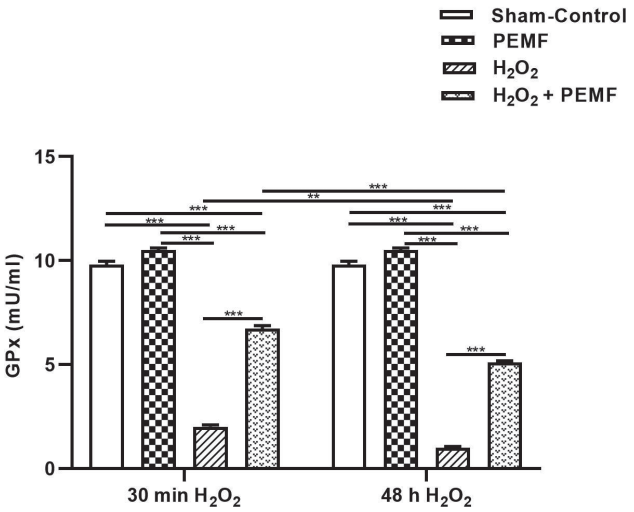

Figure 4. Comparison of the glutathione peroxidase (GPx) activity in U87-MG cell groups. Data were expressed as the mean ± SD, and were analyzed by One-Way ANOVA with Tukey's HSD *post-hoc* test. ** $p < 0.01$; *** $p \leq 0.001$. All experiments were repeated at least in triplicate. PEMF, pulsed electromagnetic field; H_2O_2, hydrogen peroxide.

of PEMF against cellular oxidative stress damage may vary depending on different time intervals of H_2O_2 treatment. ROS levels in cells that were just exposed to PEMF without H_2O_2 treatment (PEMF group) were not significantly higher as compared to sham-control group and this level not as much as higher when compared to cells treated with just H_2O_2 (H_2O_2 group) and/or exposed to PEMF after H_2O_2 treatment (H_2O_2+PEMF group). Accordingly, our results suggest that low-frequency PEMF has a potential impact on reducing ROS levels following different time intervals of H_2O_2 treatments, thus suggesting that low-frequency PEMF may have possible cytoprotective effect against deleterious effects of oxidative stress in glioblastoma cells.

Post-exposed PEMF elevates GPx activity

The GPx activity in all groups was determined spectrophotometrically and then the obtained data were examined by One-Way ANOVA in multiple comparisons with the Tukey HSD p*ost-hoc* test. The GPx activity in H_2O_2 group was significantly lower as compared to all the other groups ($p \leq 0.001$, respectively) (Fig. 4). Besides, the GPx activity following 48 h H_2O_2 treatment in H_2O_2 group were significantly lower than 30 min treatment ($p \leq 0.01$) (Fig. 4). The GPx activity becomes higher when cells exposed to PEMF after H_2O_2 treatment (H_2O_2+PEMF group) as compared to H_2O_2 group ($p \leq 0.001$, respectively) but this increase was not as much as in PEMF group when cells just exposed to PEMF without H_2O_2 treatment. Besides, in PEMF group, the GPx activity was non-significantly higher than in sham-control group which shows that PEMF has an increasing effect on antioxidant enzyme activity. When we considered time-dependent changes, the GPx activity in H_2O_2+PEMF group for 48 h H_2O_2 treatment was significantly lower than 30 min H_2O_2 treatment ($p \leq 0.001$) (Fig. 4). Overall results show that H_2O_2 treatment causes a decrease in GPx activity, whereas low-frequency PEMF exposure after H_2O_2 treatment induces an increase in GPx activity which might be related to *in vitro* cytoprotective effects of PEMF exposure. Moreover, the higher GPx activity at 30 min as compared to 48 h in H_2O_2+PEMF group that exposed to low-frequency PEMF after H_2O_2 treatment indicates that cytoprotective effects of low-frequency PEMF might be associated with time-dependent effects of stress factors such as H_2O_2 treatment.

Post-exposed PEMF increases GSH amount

The GSH amount in cell groups was measured by HPLC and One-Way ANOVA in multiple comparisons with the Tukey HSD *post-hoc* test was used to analyze the data. The GSH amount in H_2O_2 group was significantly lower than all the other groups ($p \leq 0.001$, respectively) (Fig. 5). When we considered time-dependent effect, the GSH amount in H_2O_2 group was significantly lower in 48 h as compared to 30 min treatment ($p \leq 0.01$). As seen from Figure 5, the GSH amount is high in H_2O_2+PEMF group where cells were exposed to PEMF after H_2O_2 treatment as compared to H_2O_2 group ($p \leq 0.001$, respectively). Besides, in H_2O_2+PEMF group,

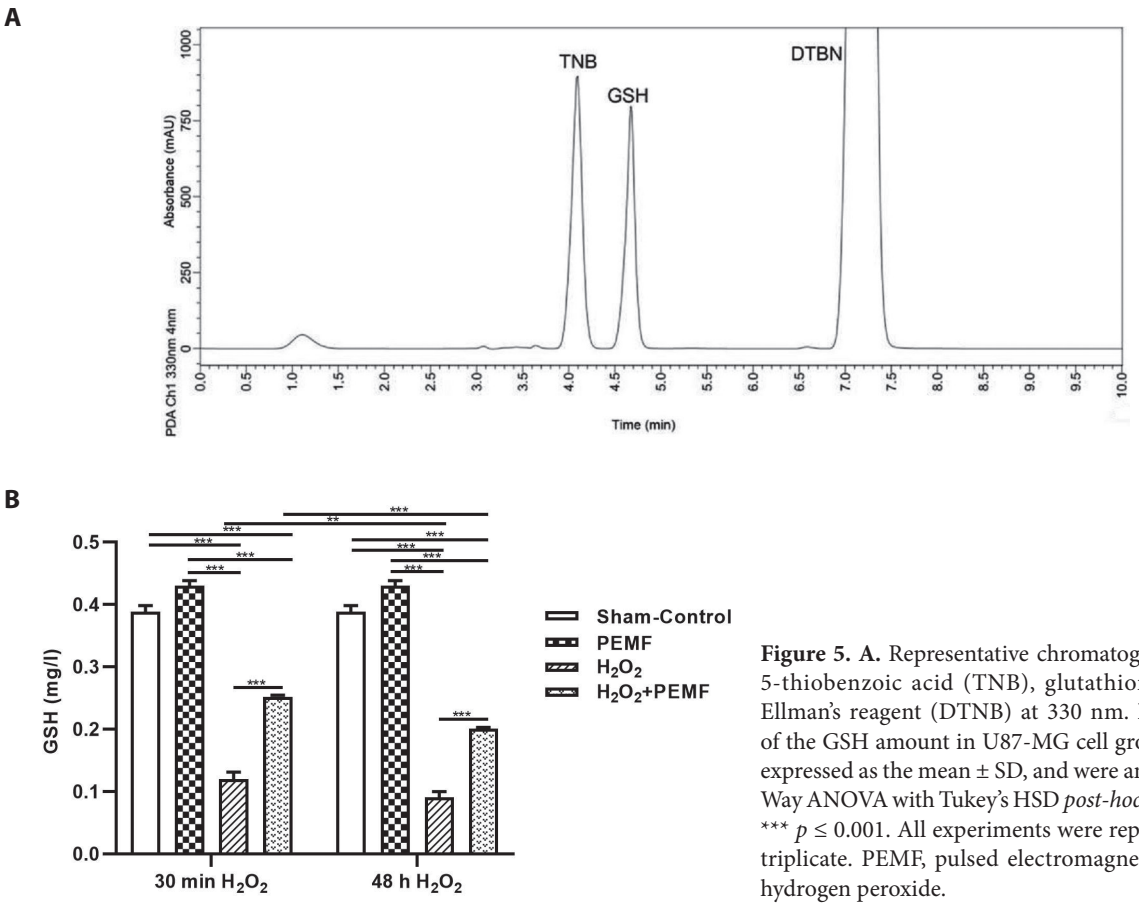

Figure 5. A. Representative chromatogram of 2-nitro-5-thiobenzoic acid (TNB), glutathione (GSH), and Ellman's reagent (DTNB) at 330 nm. **B.** Comparison of the GSH amount in U87-MG cell groups. Data were expressed as the mean ± SD, and were analyzed by One-Way ANOVA with Tukey's HSD *post-hoc* test. ** $p < 0.01$; *** $p \leq 0.001$. All experiments were repeated at least in triplicate. PEMF, pulsed electromagnetic field; H_2O_2, hydrogen peroxide.

the GSH amount following the 48 h H_2O_2 treatment was significantly lower than 30 min treatment ($p \leq 0.001$) (Fig. 5). In addition, the GSH amount was non-significantly higher in PEMF group as compared to sham-control group which indicates that PEMF has an increasing effect on antioxidant amount. Overall results indicate that H_2O_2 treatment causes a decrease in GSH amount, whereas low-frequency PEMF exposure after H_2O_2 treatment induces an increase in GSH amount. Hence, the possible cytoprotective effects of low-frequency PEMF exposure against oxidative stress in H_2O_2 treatment cells might be related to the increase in the amount of the antioxidant molecule, herein GSH. In addition, cyto-protective effects of PEMF might be correlated with time-dependent treatment of H_2O_2 since in H_2O_2+PEMF group the higher GSH amount was obtained at 30 min as compared to 48 h where cells were long time treated with H_2O_2.

Discussion

Although there are *in vivo* and/or *in vitro* controversial studies of the physiological effects of ELF-EMFs exposure, that is not deeply passed through body tissues but is absorbed by the body's surface, researchers are still interested in learning more about the underlying mechanisms and interactions between ELF-EMFs exposure and bio-systems. Among ELF-EMFs, low-frequency, low-energy PEMFs has been used as an effective, safe, and non-invasive technique in regenerative medicine to medicate several kinds of pain, such as post-operative pain (Hug and Röösli 2012; Osera et al. 2015), and in bone tissue engineering such as bone tissue repair and regeneration (Ceccarelli et al. 2013), in addition to its anti-inflammatory effects in several inflammatory disorders (Iwasa and Reddi 2018). It has also been shown that PEMFs exposure decreases hypoxia-induced cell death (Vincenzi et al. 2017; Gessi et al. 2019), reduces ischemic neuronal damage (Capone et al. 2017), enhances the antioxidant response (Falone et al. 2008, 2016; Osera et al. 2015) as well as activates the neurotrophic factors (Falone et al. 2008). Depending on the frequency, dose, exposure time, selected experimental cellular model, tissue or organism, ELF-EMFs have either cytotoxic or cytoprotective cellular effects (Akdağ et al. 2013; Vincenzi et al. 2013). In this regard, in the literature, limited studies are present showing possible effects of PEMF exposure to antioxidant defense mechanisms of neuronal cells, especially a cellular model of

human glioblastoma, U87-MG, which is an attractive model for examining the malignant and aggressive forms of astrocytic brain cancer, Glioblastoma multiforme (GBM) (Omuro and De Angelis 2013; Akbarnejad et al. 2017). Notably, there is no report showing *in vitro* effects of low-frequency PEMF post-conditioning following high-dose H_2O_2 treatment on cell viability, ROS production, GPx activity and GSH amount in U87-MG cells. Hence, in this paper, we have focused on the potential effects of 75 Hz frequency of PEMF exposure on oxidative responses in U87-MG cells. 75 Hz frequency of PEMF is considered as a standard condition to examine *in vivo* and *in vitro* effects of PEMFs by several studies as well as similar cellular responses were reported at 2 to 110 Hz PEMFs frequencies (Veronesi et al. 2014).

Oxidative stress occurs either by overproduction of reactive oxygen species (ROS) or impairment of antioxidant defense mechanism (Kang et al. 2014; Kim et al. 2015; Kıvrak et al. 2017). Glutathione (GSH) is a key cellular defense mechanism against oxidative damage and its levels in tissues are frequently utilized to assess radical damage (Kıvrak et al. 2017). In the case of oxidative stress, as the level of GSH decreases, the glutathione disulfide increases, this result in prevention of H_2O_2 accumulation and its cellular damage by the activity of GPx, which is an essential enzyme for the alleviation of oxidative stress (Zhao et al. 2001; Kıvrak et al. 2017). When the activity of GPx decreases, H_2O_2 accumulation cannot be scavenged, and in this case, free radicals generate, and the antioxidant mechanism becomes depressed (Kıvrak et al. 2017). For organisms' survival and health, a balance between the activity of these enzymes and intracellular antioxidant levels, such as GSH, is critical. In the light of these facts, changes in ROS levels, GPx activity and GSH amount among cells were focused in order to more clearly observe the effects of oxidative stress in cells.

In some tissues, EMF exposure causes oxidative stress by increasing the level of free radicals (Kıvrak et al. 2017). For example, free radical accumulation in testes (Kinnula et al. 2004), and decreasement in both SOD and GPx activities in the lung (Sepehrimanesh et al. 2016) has been reported after EMF exposure. However, to date, there is no report on effects of low-frequency PEMF to an activity of GPx and amount of GSH in human glioblastoma astrocyte-like neuronal cell line that induced oxidative stress by high-dose H_2O_2 treatment at different time intervals. For this reason, we evaluate possible changes in GPx activity and GSH amount in both H_2O_2-treated and post-exposed PEMF group following H_2O_2 treatment in addition to sham-control and PEMF-exposed groups. Hydrogen peroxide treatment in embryonic neural stem cells is known to increase mitochondrial damage, antioxidant enzymes e.g., catalase and GPx, and nitric oxide synthase activity, which points out its role in oxidative stress (Konyalioglu et al. 2013). Different from the above EMFs studies (Kinnula et al. 2004; Sepehrimanesh et al. 2016; Kıvrak et al. 2017), our results demonstrate that post-exposed PEMF increased antioxidant response by increasing GPx activity and GSH amount in U87-MG cells treated with 0.1 mM H_2O_2 at different time intervals which supports the idea that PEMF exposure has possible *in vitro* cytoprotective effects in glioblastoma cells to protect cells from damages of the experimental source of oxygen-derived free radicals, H_2O_2. Somehow similar to our study, repetitive and short-term (7 min each working day) 16 Hz PEMF effect in decreasing reactive oxygen species production by increasing GPx level and fluorescence/intracellular GSH content in human osteoclasts was documented by Ehnert et al. (2017). Herein, we propose that PEMF exposure may be a promising approach for protecting brain cancer cells by its potential ability to eliminate oxidative stress by increasing antioxidant enzyme activity and amount following free radical challenge.

Intracellularly formed hydrogen peroxide has intracellular damage by its excessive membrane permeability in various cell types, including neuronal cells, which are considered to be more susceptible to H_2O_2 toxicity (Nadin et al. 2001). Previous studies have reported that treatments of neurons to H_2O_2 with a starting concentration of 10 μM decreased survival of embryonic neural stem cells, while the concentration of 100 μM killed 45% of cells and if the concentration is 100 μM or higher, in this case, the significant decrease in neuronal cell viability produced (Konyalioglu et al. 2013; Tsai et al. 2018). Therefore, 0.1 mM of H_2O_2 concentration was selected as a high dose in the current study. We suggest that hydrogen peroxide treatment triggers oxidative stress which is supported by the increased antioxidant enzyme activity and amount. Post-PEMF exposure group following 0.1 mM H_2O_2 treatment with different time intervals has a higher percentage of cell viability than H_2O_2-treated group, indicating the cytoprotective effect of post-PEMF exposure on cell viability in U87-MG cells. This is in line with the research of Vincenzi et al. (2017), documenting the cytoprotective impact of 75 Hz short-term PEMF exposure on cell viability and apoptosis in different neuron-like cell lines including SH-SY5Y and PC12 cells in 2% O_2 hypoxic condition. The pronounced increase in cell counts, viability, and proliferation in SH-SY5Y neuroblastoma cells after 10 days of 50 Hz, 1 mT sinusoidal ELF-EMF exposure have also been reported (Sulpizio et al. 2011). Our data also indicated that the cell viability of the post-exposed PEMF group of cells was higher especially in 30 min than 48 h, which supports the idea that cytoprotective effect of short-term low-frequency PEMF exposure may vary depending on the duration of H_2O_2 treatment when the duration increases the cell viability might be decrease. This hypothesis is supported by the studies of Falone et al. (2016) and Osera et al. (2015), which report the lower number of dead SH-SY5Y and SK-N-BE(2) neuroblastoma cells were reported after

30 min H_2O_2 incubation in short and repeated pre-exposed PEMF cells compared to non-exposed cells.

Excessive production of ROS in neuronal cells, including microglial cells, causes cellular damage to proteins, lipids, and nucleic acids, leading to the death of neurons, therefore the level of ROS production is critical for neuronal survival (Vincenzi et al. 2017). The effect of low-frequency PEMF exposure on intracellular ROS levels was examined following the high-dose H_2O_2 treatment, and our results suggest that post-exposed PEMF has a reducing effect on ROS levels. This once more indicates the cytoprotective effect of short-term post-exposed low-frequency PEMF against deleterious effects of oxidative stress in glioblastoma cells. This result agrees with those found in N9 microglial cells where short-term 75 Hz PEMFs exposure reduces hypoxia-induced ROS production (Vincenzi et al. 2017). This is also supported by the studies of Osera et al. (2015) and Falone et al. (2016), showing that improving antioxidant response effect of short- and repeated PEMF pre-conditioning in drug-sensitive (SH-SY5Y) and drug-resistant (SK-N-BE(2)) human neuroblastoma cell lines against pro-oxidant challenge by restraining H_2O_2-induced ROS production and increasing MnSOD activity. Furthermore, Ehnert et al. (2017) found in human osteoclasts that ROS production was decreased by increasing the expression level of antioxidant enzymes including glutathione peroxidase 3 (GPx-3), superoxide dismutase 2 (SOD2), catalase and glutathione-s-reductase in addition to increase intracellular GSH content after repetitive short-term PEMF exposure. Similar to this work, the inhibitory effect of PEMF on intracellular ROS level was shown to inhibit osteoclastic differentiation (Pi et al. 2019). In that study, the inhibitory effect of 4 days (3 h/day) 75 Hz, 1 mT PEMF exposure on intracellular ROS generated by 1 mM H_2O_2 treatment for 30 min was documented in an osteoclast cell line (RAW264.7) with receptor activator of nuclear factor-kappa-B ligand (RANKL) (50 ng/ml) (Pi et al. 2019), which demonstrated the inhibitory effect of PEMF on RANKL-induced osteoclast differentiation by scavenging overproduction of intracellular ROS. But limited studies show possible effects of short-term 75 Hz PEMF post-conditioning following different time intervals of 0.1 mM H_2O_2 treatment on intracellular ROS levels in the neuronal cell line. Taken together, the data of our work may provide the groundwork for future experiments underlying mechanisms behind the effects of post-exposed PEMFs at different frequencies and different exposure times on to intracellular ROS levels in neuronal signaling pathways to get a more detailed conclusion.

To conclude, in the current study, it is obvious that the cytoprotective effect of post-conditioning 75 Hz, 15 min PEMF exposure on oxidative stress triggered by the different time interval of H_2O_2 treatment in U87-MG cells is mediated by the increase in the cell viability, elevation in the antioxidant enzymes' activity/amount, and the decrease in the ROS level. In light of these findings, further *in vivo* and/or *in vitro* scientific research on neurophysiological effects of PEMFs and their underlying molecular mechanisms are needed to elucidate neurotoxic or neuroprotective role against antioxidant defense mechanisms and their triggered redox-based neuronal pathways, in addition to oxidative stress-induced neurodegenerative processes. Noteworthy, PEMF parameters such as dose, frequency, intensity, amplitude, and exposure-time should be considered using different cellular, tissue, or animal models to get more robust results since such factors are critical for outcome results. Therefore, we believe that the work being done currently may shed light on further works focusing on molecular, biochemical and cellular processes activated by PEMF exposure.

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Authors' contributions. ÇGS, and SK, designed the study; ÇGS, and TŞ, performed PEMF exposure; TŞ, ÇGS, and SK, performed biochemical assays; ÇGS analyzed the data; ÇGS wrote the original draft; ÇGS coordinated the project.

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