Original Research Article

Pulsed electromagnetic field exposure reduces hypoxia and inflammation damage in neuron-like and microglial cells†

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ABSTRACT

In the present study the effect of low-frequency, low-energy pulsed electromagnetic fields (PEMFs) has been investigated by using different cell lines derived from neuron-like cells and microglial cells. In particular, the primary aim was to evaluate the effect of PEMF exposure in inflammation- and hypoxia-induced injury in two different neuronal cell models, the human neuroblastoma-derived SH-SY5Y cells and rat pheochromocytoma PC12 cells and in N9 microglial cells. In neuron-like cells, live/dead and apoptosis assays were performed in hypoxia conditions from 2 to 48 h. Interestingly, PEMF exposure counteracted hypoxia damage significantly reducing cell death and apoptosis. In the same cell lines, PEMFs inhibited the activation of the hypoxia-inducible factor 1α (HIF-1α), the master transcriptional regulator of cellular response to hypoxia. The effect of PEMF exposure on reactive oxygen species (ROS) production in both neuron-like and microglial cells was investigated considering their key role in ischemic injury. PEMFs significantly decreased hypoxia-induced ROS generation in PC12, SH-SY5Y and N9 cells after 24 or 48 h of incubation. Moreover, PEMFs were able to reduce some of the most well-known pro-inflammatory cytokines such as tumor necrosis factor -α (TNF-α), interleukin (IL)-1β, IL-6 and IL-8 release in N9 microglial cells stimulated with different concentrations of LPS for 24 or 48 h of incubation time. These results show a protective effect of PEMFs on hypoxia damage in neuron-like cells and an anti-inflammatory effect in microglial cells suggesting that PEMFs could represent a potential therapeutic approach in cerebral ischemic conditions.

Introduction

Many studies have aimed to find efficient molecules and drugs to treat ischemic stroke which is one of the most frequent causes of death and disability worldwide and has significant clinical and socioeconomic impact (Bustamante et al., 2016; Arnao et al., 2016). The pathophysiology of cerebral ischemia is complex, involving energy failure, disruption of ion homeostasis, glutamate release, calcium channel dysfunction, free radical release, mitochondrial dysfunction and membrane disruption (Maestrini et al., 2016; Chen et al., 2011). Moreover, pro-inflammatory cytokines up-regulate cell adhesion molecules and exert an important role in promoting blood cell infiltration and accumulation in ischemic tissue (Wang et al., 2007). The main risk factors for stroke are high blood pressure, tobacco smoking, obesity, high blood cholesterol, diabetes mellitus, previous transient ischemic attack and atrial fibrillation (Dyakova et al., 2016; Prabhakaran et al., 2015). Prevention includes decreasing risk factors as well as possibly aspirin, statins, surgery to open up the arteries to the brain in those with problematic narrowing, and warfarin in those with atrial fibrillation (Hahne et al., 2016; Barclay et al., 2015). Currently there is no promising pharmacotherapy for stroke aside from intravenous or intra-arterial thrombolysis or a mechanical embolus removal (Holodinsky et al., 2016).

Electromagnetic fields are emerging as a potential alternative to the pharmacological treatments in several inflammatory related pathologies. Previous studies have tried to clarify the mechanisms of interaction between low frequency electromagnetic fields and biological systems (Hardell et al., 2008; Capone et al., 2009; Di Lazzaro et al., 2013a). In vitro experiments in various types of cells and tissues have suggested that physiological systems can be influenced by electromagnetic field exposure (Massot et al., 2000; Varani et a., 2012). It has been reported a protective effect of electromagnetic field exposure in animal models of neurodegenerative diseases as Alzheimer's and Parkinson's diseases (Arendash et al., 2010; Wang et al., 2010). In addition, several papers are present in literature on the in vitro and/or in vivo effects of the low-frequency,

low energy pulsed electromagnetic fields (PEMFs). In particular, various studies on cancer have shown that electromagnetic fields reduce the tumor growth and proliferation (Cameron et al., 2005; Barbault et al., 2009; Jiménez-García et al., 2010). It has been also reported that PEMFs in various tumor cells are able to reduce NF-kB stimulation, cell proliferation and to increase p53 activation, cytotoxicity and apoptosis (Vincenzi et al., 2012). Moreover, PEMF therapy significantly reduced post-operative pain and narcotic use in the immediate post-operative period by a mechanism that involve endogenous interleukin-1β (IL-1β) in the wound bed (Rohde et al., 2010). Several papers have demonstrated the anti-inflammatory effect of PEMF exposure in human synoviocytes, chondrocytes and osteoblasts with a significant reduction of some of the most relevant pro-inflammatory cytokines (Varani et al., 2008; Ongaro et al., 2012; Vincenzi et al., 2013). According to recent literature, it has been reported a beneficial effect of electromagnetic fields on hypoxia-related conditions. The PEMF exposure inhibited hypoxia/reoxygenation-induced death of human renal proximal tubular cells via suppression of intracellular reactive oxygen species (ROS) production (Lim et al., 2015). Moreover, it has been demonstrated that electromagnetic fields when applied prior to, during, and after the ischemic insult, protects the heart against ischemia/reperfusion-induced cardiac contractile dysfunction and heart injury (Bialy et al., 2015). In a murine model of hindlimb ischemia, PEMF exposure improved ischemia-induced angiogenesis through enhancing endothelial proliferation, migration, survival and secretion via acting on the Akt-eNOS-VEGF pathway of endothelial cells (Li et al., 2015). In addition, the protective effects of PEMFs have been demonstrated in cardiomyocytes against hypoxia-induced injury ameliorating intracellular Ca^{2+} homeostasis via heat shock protein 70 activation (Wei et al., 2016). In vivo studies have demonstrated that electromagnetic stimulation may accelerate the healing of tissue damage following ischemia suggesting that the exposure to a PEMF of short duration may have implications for the treatment of acute stroke (Grant et al., 1994). In a distal middle cerebral artery occlusion in mice, PEMF significantly influenced expression profile of pro- and anti-inflammatory factors in the hemisphere ipsilateral to

ischemic damage (Pena-Philippides et al., 2014). In the same experimental model of cerebral ischemia it has been also observed a significant reduction of infarct size mediated by a chronic treatment of PEMFs as compared to controls (Pena-Philippides et al., 2014).

From this background it is evident that limited results are present in literature on the PEMF effect in neuronal cells following hypoxia injury. A cellular model represented by a human neuroblastoma cell line, SH-SY5Y appears to be an attractive system for studying neuron-like cells (Vekrellis et al., 2009). Another cellular line well used as a model of neuronal cells is represented by rat pheochromocytoma PC12 cells (Vincenzi et al., 2012). Inflammation plays a critical role in mediating post-ischemic injury and the activation of microglia, the major resident immune cells in the brain, is a key element in triggering the innate immune response. N9 cell line is a commonly used model to study inflammatory responses of microglial cells (Corradin et al., 1993). In the current study the effect of PEMFs has been investigated by using different cell lines derived from neuron-like cells such as SH-SY5Y and PC12 cells. In particular, the protective effect of PEMFs on cell viability and on apoptosis in normoxic or hypoxic conditions has been found. To investigate the cellular mechanism of PEMFs, hypoxia inducible factor 1α (HIF-1α) activation and ROS production has been studied in normoxic or hypoxic conditions suggesting their partial restore after PEMF treatment. Moreover, in N9 microglial cells, PEMF exposure mediates a significant reduction of ROS production and of some of the most relevant pro-inflammatory cytokines.

Materials and Methods

Cell cultures

Rat pheochromocytoma (PC12) cells were purchased from American Type Culture Collection (Manassas, VA, USA) and were maintained in DMEM F12 medium (Invitrogen, Grand Island, NY, USA) supplemented with 5% FBS (Thermo Scientific, Waltham, MA, USA), 10% horse serum, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified atmosphere (5% CO_2) at 37°C. Cells were subcultured three times a week at a density of

500000/ml and the differentiation was achieved by treatment with 50 ng/ml nerve growth factor (NGF, Sigma, St Louis, MO, USA) for one week (Barbault et al., 2009). Cells were cultured in DMEM medium (Invitrogen, USA) supplemented with 10% FBS (Thermo Scientific, USA) and the cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 (Reale et al., 2014). The N9 murine microglial cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Corradin et al., 1993). Cells were grown in a humidified environment containing 5% CO_2 at a constant temperature of 37°C.

Before the experiments, the cell culture medium was replaced with fresh serum-free medium for another 24 h to minimize the interference of growth factors in the serum with signal transduction.

Electromagnetic Field Exposure System

The neuronal-like and microglial cells were exposed to PEMFs generated by a pair of rectangular horizontal coils (14 cm × 23 cm), each made of 1400 turns of copper wire placed opposite to each other. The complete exposure system has been previously described in detail (Cadossi et al., 1992; Varani et al., 2012). The culture was placed between this pair of coils so that the plane of the coils was perpendicular to the culture flasks. The coils were powered by the PEMF generator system (IGEA, Carpi, Italy) used in previous studies (Massot et al., 2000; Barbault et al., 2009; Varani et al., 2008; Ongaro et al., 2012; Vincenzi et al., 2013; Varani et al., 2002; Varani et al., 2003; De Mattei et al., 2009; Fini et al., 2013), which produced a pulsed signal with the following parameters: pulse duration of 1.3 ms and frequency of 75 Hz, yielding a 0.1 duty cycle. The peak intensity of the magnetic field and peak intensity of the induced electric voltage were detected in air between two coils from one side to the other, at the level of the culture flasks. The peak values measured between two coils in air had a maximum variation of 1% in the whole area in which the culture flasks were placed. The dimensions of the flasks were 9.2 cm × 8.2 cm with 10 ml of medium. The peak intensity of the magnetic field was 1.5 ± 0.2 mT and it was detected using the

Hall probe (HTD61-0608-05-T, F.W. Bell, Sypris Solutions, Louisville, KY) of a gaussmeter (DG500, Laboratorio Elettrofisico, Milan, Italy) with a reading sensitivity of 0.2%. The corresponding peak amplitude of the induced electric voltage was 2.0 ± 0.5 mV. It was detected using a standard coil probe (50 turns, 0.5 cm internal diameter of the coil probe, 0.2 mm copper diameter) and the temporal pattern of the signal was displayed using a digital oscilloscope (Le Croy, Chestnut Ridge, NY). The shape of the induced electric voltage and its impulse length were kept constant. A photograph of the PEMF exposure system used in the experimental assays is depicted in Fig.1.

Analysis of cell viability

Cell viability was investigated by using the Live and Dead Cell Assay (Abcam, Cambridge, UK). Live cells were identified on the basis of intracellular esterase activity (generating green fluorescence) and exclusion of the red dye. Dead cells were identified by the lack esterase activity and non-intact plasma membrane which allows red dye staining. Cells were labelled with the Live and Dead Dye and analysed by using a Nikon fluorescent microscope (Eclipse 50i) with emission of 495 nm and excitation of 515 nm for live cells or emission of 528 nM and excitation of 617 nm for dead cells (Sun et al., 2014).

Apoptosis Assay

Apoptosis assay was performed evaluating active caspase-3 levels after different times of incubation in normoxia or hypoxia in the absence or in the presence of PEMF exposure. At the end of each incubation time, the cells were treated with biotin-ZVKD-fmk inhibitor (10 µM) for 1 h at room temperature. After discarding the culture media, cells were rinsed with PBS and the extraction buffer containing protease inhibitors was added to prepare cell extracts. After 2 h of incubation at room temperature 100 µl of samples were transferred into a microplate pre-coated with a monoclonal antibody specific for caspase-3. After washing, 100 µl of streptavidin caspase-

3 conjugated to horseradish peroxidases that binds to the biotin of the inhibitor were added. Following the wash, the substrate solution was added to the wells for 30 min and stop solution was used to block the reaction. The optical density was determined using a microplate reader set to 450 nm (Varani et al., 2011).

HIF-1α analysis

For HIF-1α detection, the cells were put under normoxia or hypoxia. Nuclear extracts from PC12 and SH-SY5Y cells were obtained by using a nuclear extract kit (Abcam, Cambridge, UK) according to the manufacturer instructions. HIF-1α activation was evaluated by using HIF-1α Transcription Factor Assay (Abcam, Cambridge, UK). HIF-1α specifically binds to the immobilized double stranded DNA (dsDNA) sequence containing the HIF-1α response element (5'-ACGTG-3'). The HIF-1α transcription factor complex was detected by addition of a specific primary antibody. A horseradish peroxidase (HRP)-conjugated secondary antibody was added to provide a sensitive colorimetric readout that was quantified by spectrophotometry at 450 nm wavelength (Merighi et al., 2015).

ROS production

The production of ROS was evaluated with a DCFDA cellular ROS detection assay (Abcam, Cambridge, UK). Cells were seeded in a 96 well plate and, after stimulation with hypoxia or LPS in the absence or in the presence of PEMFs, a solution of DCFDA was added. Fluorescence was measured in a EnSight Multimode Plate Reader (Perkin Elmer, Boston, MA, USA) with an excitation at 485 nm and an emission at 535 nm (Lunov et al., 2014).

Pro-inflammatory cytokine release

The cells were suspended at a density of 10^6 cells/ml and seeded into 24-well plates. Cells were incubated for 24 or 48 h in the absence or in the presence of lipopolysaccharide (LPS, 0.1, 0.5 or 1

μg/ml). At the end of incubation, the cell suspension was collected and centrifuged at 1000 x g for 10 min at 4°C. The pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8 levels were determined with specific quantitative sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer instructions. The reaction was developed with streptavidin-horseradish peroxidase and optical density was read at 450 nm wavelength (Vincenzi et al., 2013).

Data and statistical analysis

Analysis of data was performed by one-way analysis of variance followed by Bonferroni post-hoc comparisons test to test inter-group differences. Differences between the groups were considered significant at a value of $P<0.01$ by using Graph Pad Prism software (version 6.0 GraphPad Software, Inc, San Diego, CA). All experimental data are reported as mean ± SEM from at least 6 independent experiments.

Results

PEMF exposure decreases hypoxia-induced neuron-like cell death

The incubation of PC12 and SH-SY5Y cells in hypoxic conditions (2% O_2) from 2 to 48 h determined a time-dependent increase in the number of dead cells in comparison to normoxic condition (Fig. 2). The simultaneous exposure of PC12 cells to PEMFs in hypoxic condition resulted in a significant reduction of the percentage dead cells after 6, 12, 24 and 48 h of incubation (Fig. 2). Similar results were also obtained in SH-SY5Y cells where the presence of PEMFs significantly counteracted the hypoxia-induced cell death from 6 to 48 h of incubation (Fig. 2). A representative microphotograph of PC12 and SH-SY5Y cells after 24 h of normoxia, hypoxia and hypoxia in the presence of PEMFs where it is evident a reduction of dead cells (red stained) after PEMF exposure in comparison to hypoxic condition (Fig. 3).

Moreover the effect of PEMFs on apoptotic signals was evaluated by using the levels of active caspase-3 from 2 to 48 h of incubation (Fig. 4). Among the different treatment conditions, only

hypoxia after 12 h mediated a significant increase of the levels of active caspase-3 in both the cells examined (Fig. 4). Furthermore, PEMF exposure reduced the pro-apoptotic effect of hypoxia after 12, 24 and 48 h of incubation (Fig. 4).

PEMFs reduces hypoxia-stimulated HIF-1α levels in PC12 and SH-SY5Y cells

To shed some light on the mechanism of action of PEMFs in the surviving of neuron-like cells, the modulation of the transcription factor HIF-1α was investigated. HIF-1α is a key regulator in hypoxia and an important player in neurological outcomes following ischemic stroke due to the functions of its downstream genes. Fig. 5 reports HIF-1α activation after 2, 4 or 6 h of incubation with PEMFs in normoxic or hypoxic conditions. As expected, under hypoxic conditions a 4-fold increase of HIF-1α levels has been found respect to control condition. The PEMF treatment (4 h) mediated a significant reduction of HIF-1α expression (25% respect to hypoxia) suggesting a partial recovery versus the normoxic condition.

PEMFs reduces hypoxia-stimulated ROS levels in neuron-like and microglial cells

The effect of PEMFs in PC12 and in SH-SY5Y cells was also investigated on ROS production as an important mediator of ischemic damage (Fig. 6A and 6B, respectively). In both examined cells, as reported in Fig. 6, hypoxia mediated an increase of ROS production whilst PEMF exposure resulted in a significant reduction of hypoxia-stimulated ROS formation. Similarly, in N9 cells, PEMFs were able to significantly reduce ROS production after 24 h and 48 h of incubation (Fig. 6C).

PEMFs reduces pro-inflammatory cytokines in N9 microglial cells

As expected LPS stimulation at different concentrations (from 0.1 to 1 μg/ml) was able to activate N9 microglial cells mediating a significant increase of TNF-α, IL-1β, IL-6 and IL-8 production in the absence or in the presence of PEMFs after 24 or 48 h of incubation (Fig. 7 and 8). Interestingly

PEMF exposure was able to significantly reduce the LPS-stimulated TNF-α production (Fig. 7A, 7B). In N9 cells, the presence of PEMFs also mediated a significant reduction of IL-1β production (Fig. 7C, 7D). The effect of LPS stimulation on IL-6 and IL-8 production was also investigated showing a dose-dependent release of these pro-inflammatory cytokines (Fig. 8). In N9 cells, PEMF exposure was able to significantly reduce the LPS-stimulated IL-6 and IL-8 production after 24 or 48 h of incubation suggesting a direct anti-inflammatory effect (Fig. 8).

Discussion

Previous studies show that PEMFs could be considered a viable therapeutic approach used to control inflammation that is associated with different diseases (Massari et al., 2006; Zorzi et al., 2007; Benazzo et al., 2008; Cadossi, 2011; Ongaro et al., 2011; Di Lazzaro et al., 2013b; Di Lazzaro et al., 2016). It has been well reported that the cellular effects of PEMFs depend on their intensity and exposure time as well as on the cellular phenotype and interaction with intracellular structures (Massot et al., 2000; Barbault et al., 2009; Varani et al., 2008; Ongaro et al., 2012; Vincenzi et al., 2013; Varani et al., 2002; Varani et al., 2003; De Mattei et al., 2009; Fini et al., 2013). Several papers are present in the literature reporting a frequency of 75 Hz as a standard condition to evaluate the in vitro and in vivo effects of PEMFs (Varani et al., 2012, Veronesi et al., 2014, Massari et al., 2006). On the other hand, it has been showed that the use of PEMFs at different frequencies (from 2 to 110 Hz) induced similar cellular responses (De Mattei et al., 2007). From the clinical point of view the evaluation of PEMF effect on ischemic stroke as potential effective tool to promote recovery in acute ischemic stroke patients is currently in progress (Di Lazzaro et al., 2013b; Di Lazzaro et al., 2016).

In this paper we have investigated the potential effect of PEMFs in hypoxic condition by using PC12 and SH-SY5Y cells. Both these cell lines are able to proliferate in vitro for long periods and represent generally accepted experimental models for neurological studies involving neuronal metabolism and neuroadaptive processes (Xie et al., 2010). In PC12 and SH-SY5Y cells, PEMF exposure significantly reduced hypoxia-induced cell death. In order to evaluate if the observed

effect of death also involved apoptotic events, active caspase-3 levels were investigated in the cell line examined following the hypoxia treatment in the presence or in the absence of PEMF exposure.

Interestingly, the hypoxic conditions were able to increase both cell death and the caspase-3 levels, effects that were significantly decreased by PEMF exposure, suggesting the important role of the PEMFs in the induction of cell death and apoptosis. This protective effect was evident incubating the cells with PEMFs simultaneously to hypoxia as well as exposing the cells to PEMFs after 6 h of hypoxic condition. These data are in agreement with those obtained in vivo where PEMF exposure attenuated cortical ischemia edema and reduced ischemic neuronal damage in a rabbit model of transient focal ischemia (Grant et al., 1994). Moreover, in a distal middle cerebral artery occlusion model in mice, the infarct size was significantly smaller in PEMF-treated animals as compared to controls (Pena-Philippides et al., 2014). A direct effect of electromagnetic fields has been also reported in neuronal cell models where they increased differentiation, neurite outgrowth and survival (Podda et al., 2014; Lekhraj et al., 2014; Ma et al., 2016).

To better understand the mechanisms underlying their protective effect, we evaluated whether PEMFs could affect the expression of HIF-1α, a master regulator of the cellular response to hypoxia. Hypoxia-inducible factors mediate adaptive responses to ischemia by induction of anti- and pro-survival genes (Semenza, 2012). Recently, it has been reported that neuronal HIF-1α and HIF-2α deficiency improves neuronal survival in the early acute phase after ischemic stroke (Barteczek et al., 2016). Moreover, excessive HIF-1α activation has been associated to neuronal apoptosis in different cellular models of brain injury (Long et al., 2014; Huang et al., 2016; Cheng et al., 2014). In the present paper we have found that PEMFs exposure in PC12 and SH-SY5Y cells partially reduced hypoxia-induced HIF-1α activation. From these data, we can speculate that PEMFs are able to counteract the excessive activation of HIF-1α without completely block its expression that could be considered protective for the cell survival. As a matter of fact, it is well

recognised that under hypoxic condition HIF-1α subunit is involved in different cellular responses that promote cell survival (Semenza, 2011a; Semenza, 2011b).

It is becoming clear that a common denominator in diverse pathogenic mechanisms is oxidative stress accompanied by redox dysregulation which have a role in metabolic and mitochondrial dysfunction and excitoxicity (Espinosa-Diez, et al., 2015). In ischemic condition, ROS production is a critical event that damage cellular proteins, lipids and nucleic acids leading to neuronal death (Sanderson et al., 2013). In our experiments, the exposure of PC12 and SH-SY5Y cells to PEMFs improved cellular response against a noxious insult such as hypoxia mainly through a ROS level reduction. These data are in agreement with those found in human neuroblastoma cell line where PEMFs prevents H_2O_2-induced ROS production by increasing superoxide dismutase activity (Osera et al., 2015). These data might suggest that ROS-targeting defence mechanisms are activated by PEMFs and that this activation could counteract the hypoxic insult. The significant reduction on HIF-1α activation and ROS production by PEMF exposure in PC12 and SH-SY5Y cells could explain the protective effect of PEMFs on hypoxia-induced cell death.

It is well recognized that excessive ROS generation not only is detrimental for neuronal survival during ischemia, but could also activate inflammatory cascade in microglial cells. Endogenous microglia represent a network of immunocompetent cells highly responsive to environmental stress and immunological challenges (Salter and Beggs, 2014). Microglial cells are activated following cerebral ischemia playing a crucial role in neuroinflammation mainly releasing proinflammatory cytokines such as TNF-α and IL-1β (Amantea et al., 2015). We used N9 cells to investigate the influence of PEMF exposure on oxidative and inflammatory responses in microglial cells. The results clearly indicated that PEMFs were able to significantly decrease hypoxia-induced ROS production in N9 microglial cells. In the same cell line PEMF exposure reduced some of the most important pro-inflammatory cytokine such as TNF-α, IL-1β, IL-6 and IL-8. For the studies on cytokine release, we have stimulated N9 microglial cells with LPS, that represent a commonly used inflammatory factor. Since inflammation is a consequent response to

ischemic injury, the evaluation of the effect of PEMFs on the release of LPS-stimulated cytokines from microglial cells strengthen the potential therapeutic of PEMFs for hypoxic damage. It is well known that the cytokines are upregulated in the brain after a variety of insults including stroke and are expressed not only in cells of the immune system but in resident brain cells including microglia (Wang et al., 2007). Among these cytokines, IL-1β and TNF-α appear to exacerbate cerebral injury and their inhibition reduced ischemic brain damage (Allan and Rothwell, 2001).

In conclusion, these data showed a direct protective effect of PEMF exposure in PC12 and SH-SY5Y subjected to hypoxic insult. In these neuron-like cells, PEMFs were able to partially restore HIF-1α activation and to inhibit ROS production following hypoxic incubation. In N9 microglial cells PEMFs exposure significantly reduced ROS generation and pro-inflammatory cytokine release, crucial events in the exacerbation of ischemic condition. These results indicate the possibility that a non-invasive stimulus represented by PEMFs could have a potentially important positive impact on the post-stroke recovery process, implicating PEMF as a possible adjunctive therapy for stroke patients.

Acknowledgements

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Figure Legends

Fig. 1. Photograph of the PEMF exposure system used.

Fig. 2. PEMF exposure reduced hypoxia-induced cell death in PC12 cells (A) and SH-SY5Y cells (B). Histogram relative to the Live/Dead assay after 2, 4, 6, 12, 24 and 48 h of incubation in normoxia, hypoxia and hypoxia in the presence of PEMFs. n = 6 for each condition. *, $P<0.01$ vs normoxia, **, $P<0.01$ vs hypoxia.

Fig. 3. Protective effect of PEMFs in neuron-like cells. Representative microphotograph of PC12 (A,B,C) and SH-SY5Y (D,E,F) cells after 24 h of incubation in normoxia (A,D), hypoxia (B,E) and hypoxia in the presence of PEMFs (C,F).

Fig. 4. PEMF exposure reduced hypoxia-induced apoptosis in PC12 cells (A) and SH-SY5Y cells (B). Histogram relative to the active caspase-3 levels after 2, 4, 6, 12, 24 and 48 h of incubation in normoxia, hypoxia and hypoxia in the presence of PEMFs. n = 8 for each condition. *, $P<0.01$ vs normoxia, **, $P<0.01$ vs hypoxia.

Fig. 5. PEMFs partially decreased hypoxia-stimulated HIF-1α activation. HIF-1α activation in PC12 (A) and SH-SY5Y (B) cells after 2, 4 and 6 h of incubation in hypoxia in the absence or in the presence of PEMFs. n = 8 for each condition. *, $P<0.01$ vs normoxia; **, $P<0.01$ vs hypoxia.

Fig. 6. PEMF exposure reduced hypoxia-stimulated ROS production in PC 12 (A), SH-SY5Y (B) and N9 (C) cells. ROS production was evaluated after 24 or 48 h of hypoxia in the absence or in the presence of PEMFs. n = 6 for each condition. *, $P<0.01$ vs normoxia; **, $P<0.01$ vs hypoxia.

Fig. 7. PEMF exposure reduced LPS-induced TNF-α or IL-1β release in N9 microglial cells. TNF-α (A,B) or IL-1β (C,D) levels were measured after 24 (A,C) or 48 (B,D) h of LPS stimulation in the absence or in the presence of PEMFs. n = 8 for each condition. *, $P<0.01$ vs control; **, $P<0.01$ vs LPS.

Fig. 8. PEMF exposure reduced LPS-induced IL-6 or IL-8 release in N9 microglial cells. IL-6 (A,B) or IL-8 (C,D) levels were measured after 24 (A,C) or 48 (B,D) h of LPS stimulation in the

absence or in the presence of PEMFs. n = 8 for each condition. *, $P<0.01$ vs control; **, $P<0.01$ vs LPS.

Figure 1

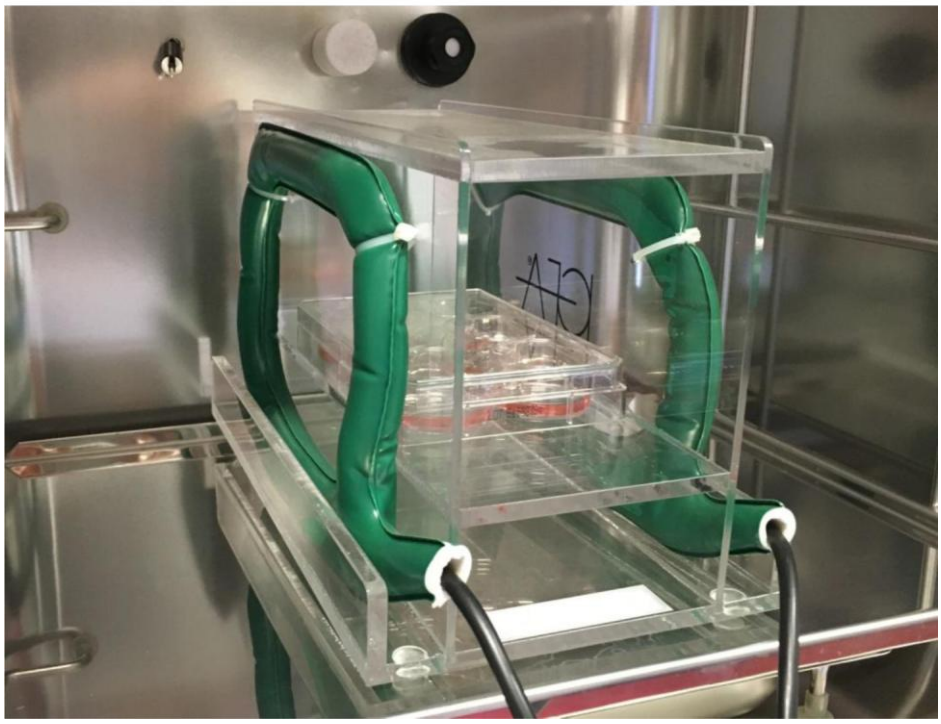
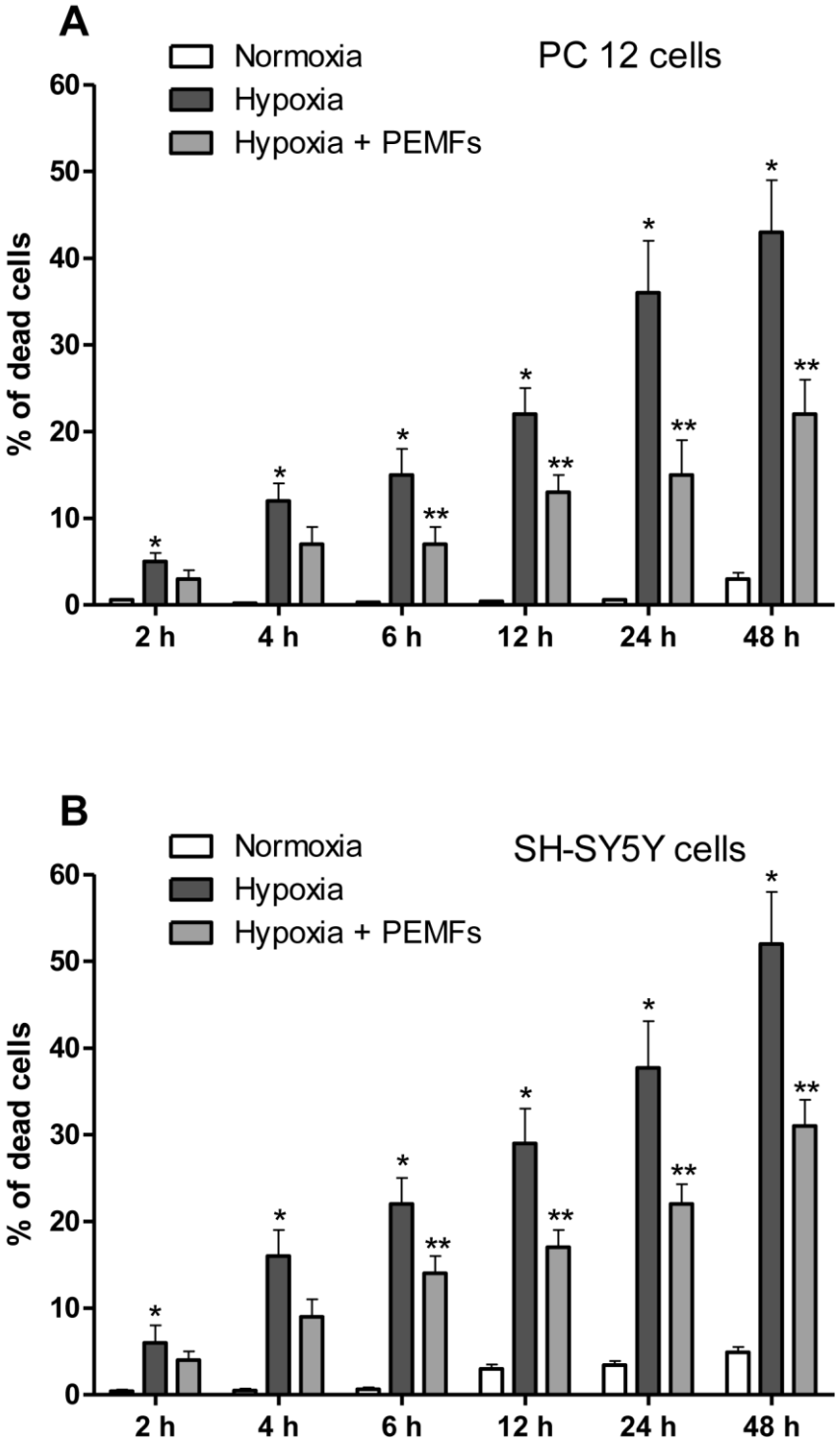


Figure 2



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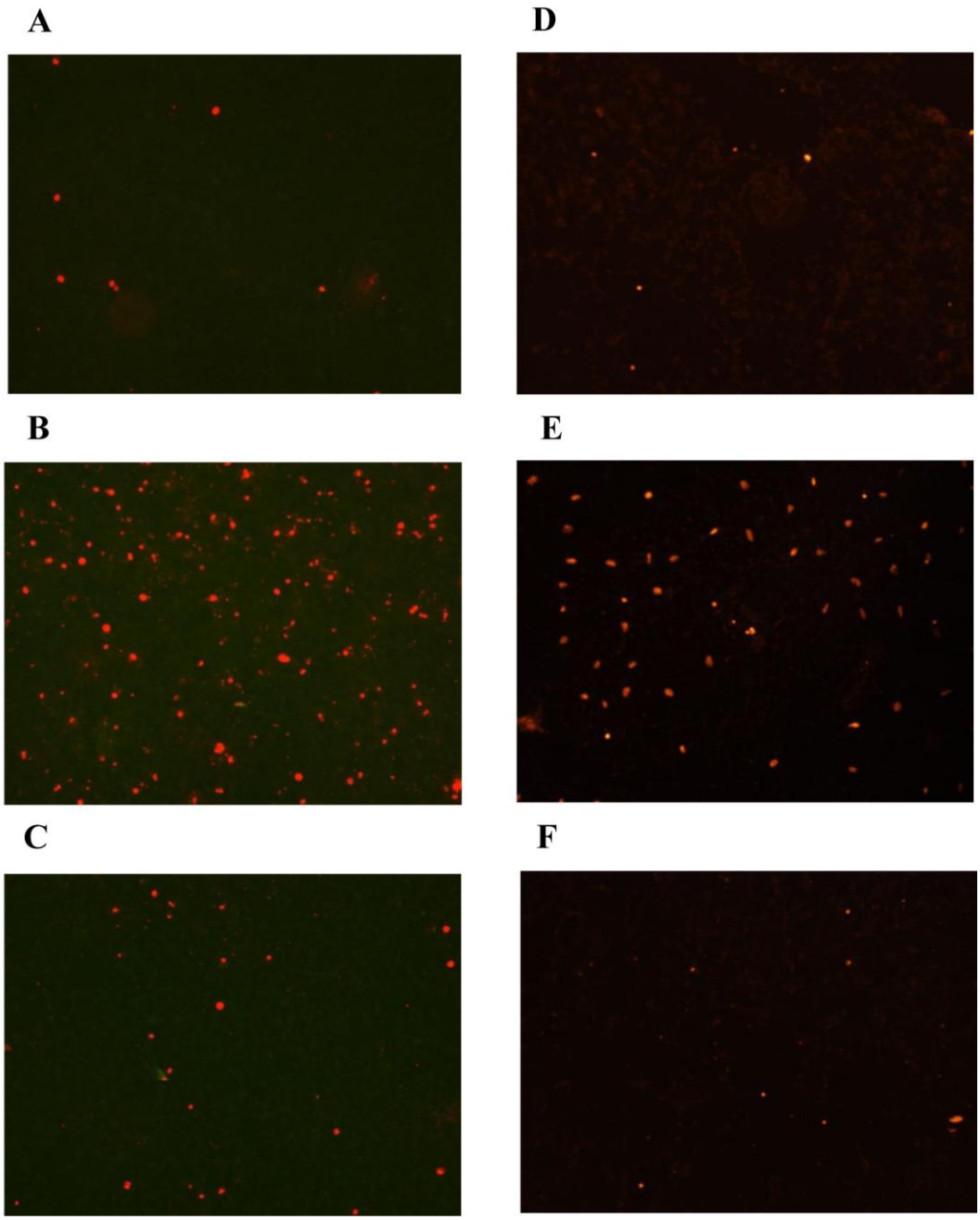

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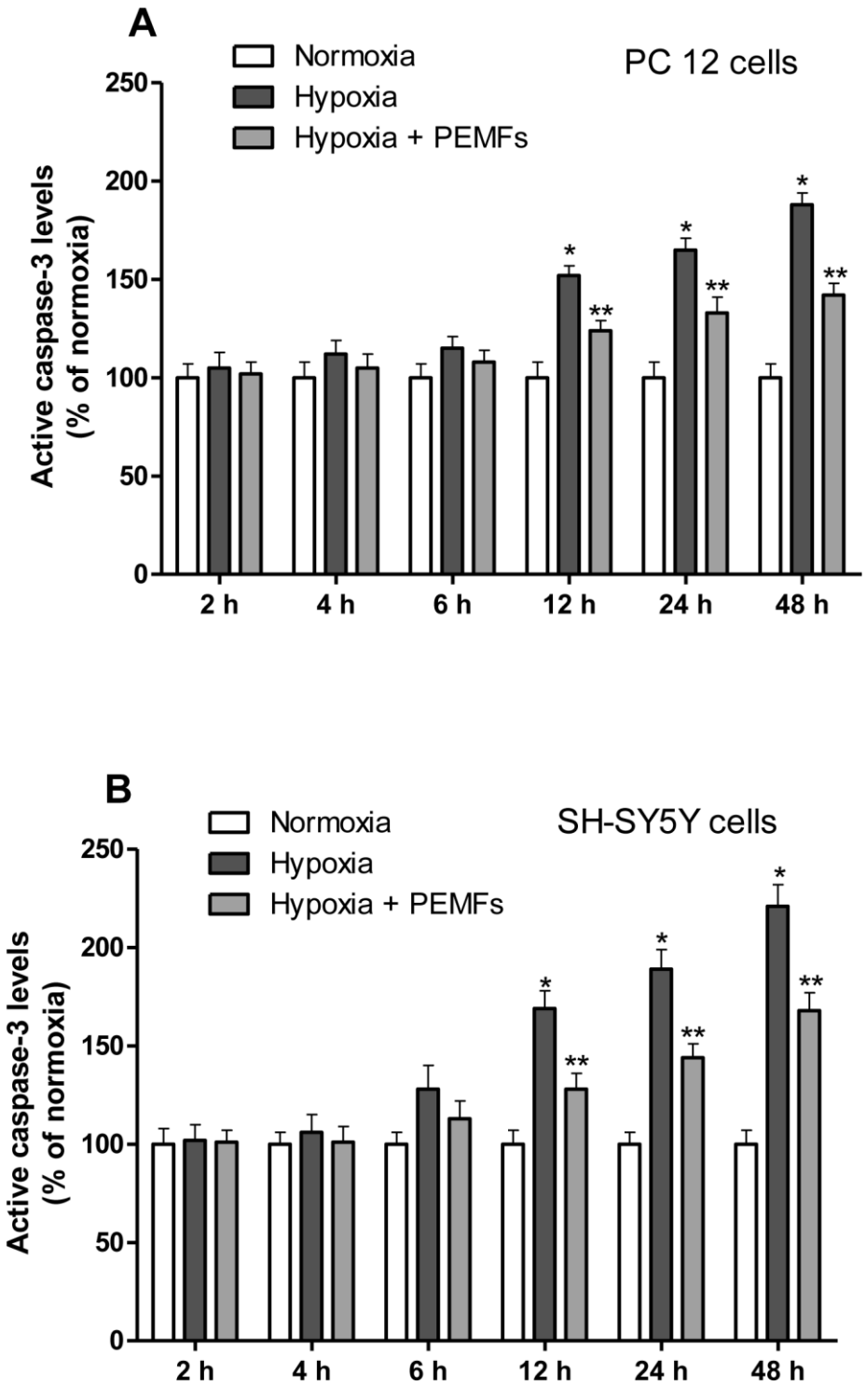

Figure 5

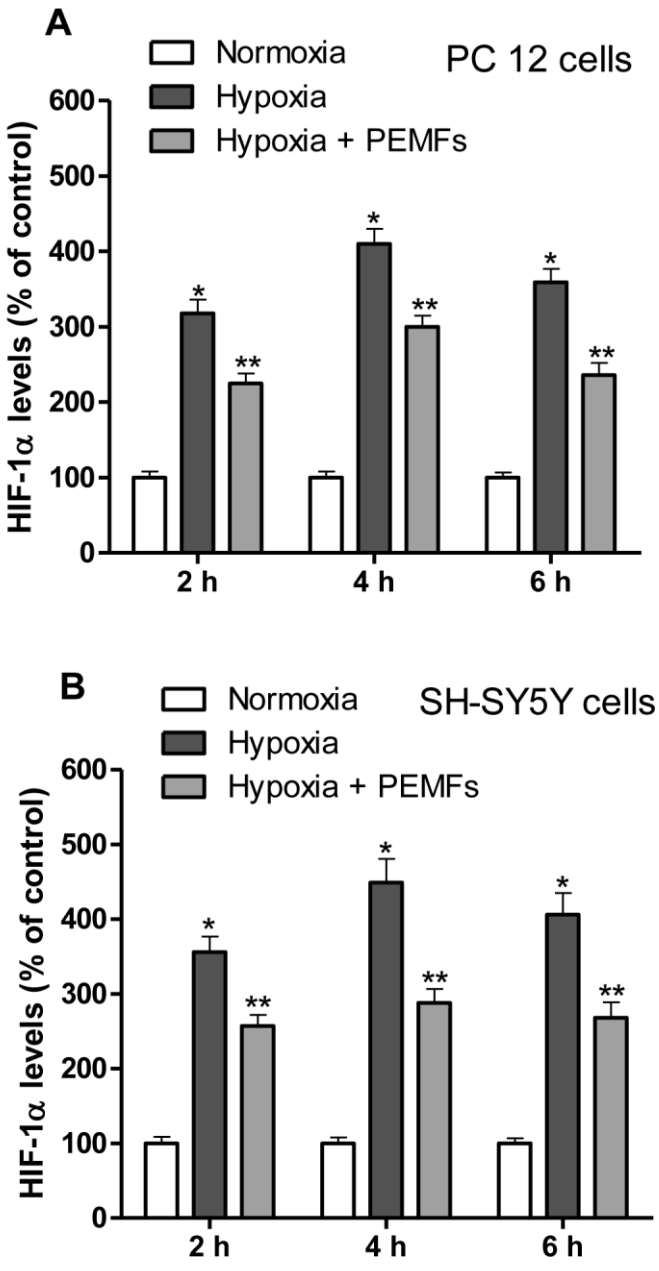

Figure 6

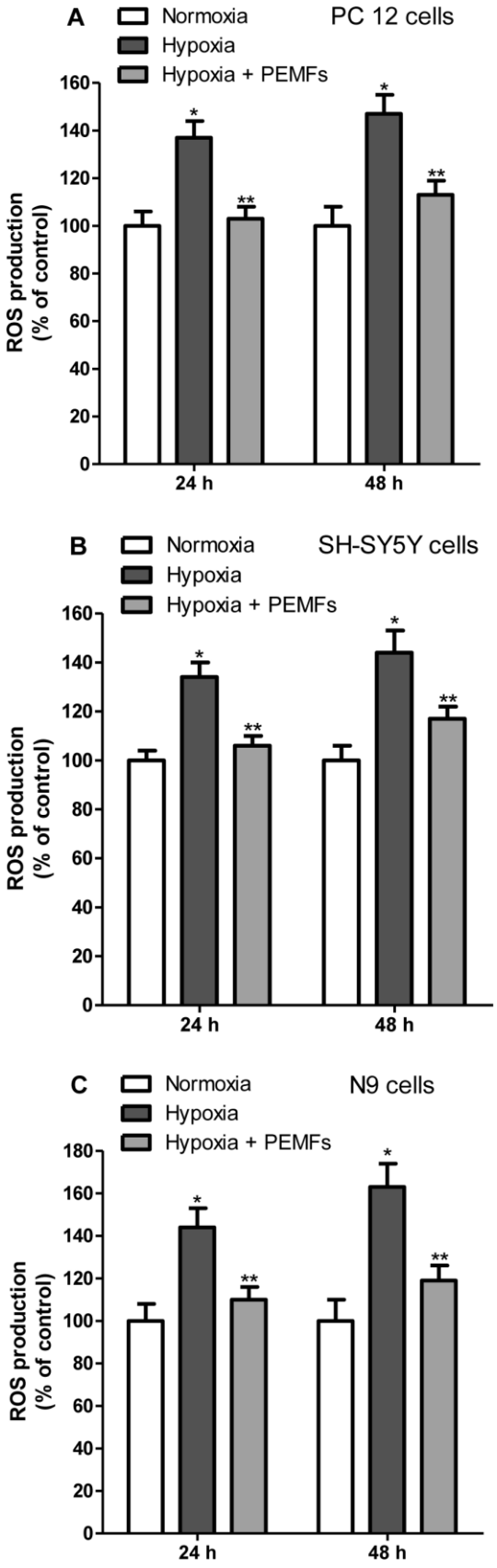

Figure 7

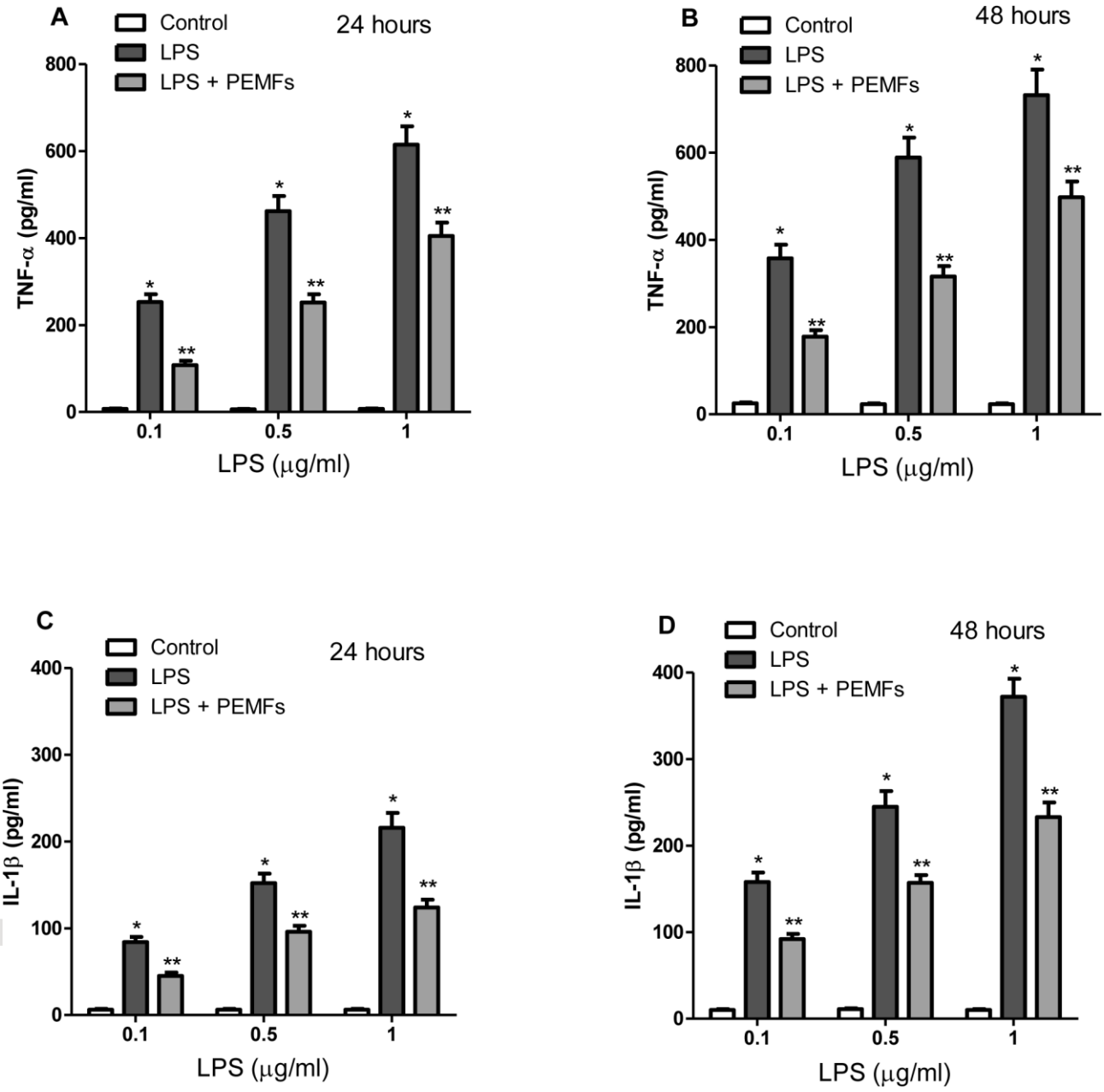

Figure 8