Pulsed electromagnetic field and relief of hypoxia-induced neuronal cell death: the signalling pathway

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Abstract

Low-energy low-frequency pulsed electromagnetic fields (PEMFs) exert several protective effects, such as regulation of kinases, transcription factors as well as cell viability in both central and peripheral biological systems. However, it is not clear on which bases they affect neuroprotection and the mechanism responsible is yet unknown.

In this work we have characterized in nerve growth factor (NGF)-differentiated pheochromocytoma PC12 cells injured with hypoxia: i) the effects of PEMF exposure on cell vitality; ii) the protective pathways activated by PEMFs to relief neuronal cell death, including adenylyl cyclase (AC), phospholipase C (PLC), protein kinase C epsilon (PKC-ε) and delta (PKC-δ), p38, ERK1/2, JNK1/2 mitogen activated protein kinases (MAPK), Akt and Caspase-3; iii) the regulation by PEMFs of prosurvival HSP70, CREB, BDNF and Bcl-2 family proteins.

The results obtained in this study show a protective effect of PEMFs that are able to reduce neuronal cell death induced by hypoxia by modulating p38, HSP70, CREB, BDNF and Bcl-2 family proteins. Specifically, we found a rapid activation (30 min) of p38 kinase cascade, which in turns enrolles HSP70 survival chaperone molecule, resulting in a significant CREB phosphorylation increase (24 h). In this cascade, later (48 h), BDNF and the antiapoptotic pathway regulated by the Bcl-2 family of proteins are recruited by PEMFs to enhance neuronal survival.

This study paves the way to elucidate the mechanisms triggered by PEMFs to act as new neuroprotective approach to treat cerebral ischemia by reducing neuronal cell death.

Introduction

Pulsed electromagnetic fields (PEMFs) therapy, a non-invasive, safe and effective method approved by the US Food and Drug Administration for treating delayed union or non-union fractures, affects physiological systems by delivering an inductive signal coupled to the treatment site, thus promoting anti-inflammatory and pro-regenerative events in mammals. Specifically, many studies demonstrate their beneficial effects in the treatment of a variety of inflammatory disorders including pseudoartrosis, joint disease, complications related to diabetes mellitus, impaired wound healing, pain and neurodegenerative pathologies (Cebrian et al., 2010; Jing et al., 2011; Pan et al., 2013; Weintraub et al., 2009; Canedo-Dorantes et al., 2002; Roland et al., 2000; Goudarzi et al., 2010; Heden et al 2008; Varani et al., 2012; 2017; Iwasa and Reddi, 2018).

Interestingly, PEMFs have been reported as neuroprotective tools due to their antiapoptotic effect against ischemic cell death, thus emerging as a potential alternative to the pharmacological protocols in ischemic diseases (Grant et al., 1994; Pena-Philippides et al., 2014; Li et al., 2015). This finding together with other important evidences obtained in animal models of cerebral ischemia, have been the basis to introduce PEMFs treatment in a clinical multicentre, prospective, randomized, placebo-controlled, double-blind study to investigate the effectiveness of PEMFs in acute ischemic stroke, as non-invasive and safe tool to gain neuron recovery (Pena-Philippides et al., 2014; Grant et al., 1994; Capone et al., 2017; NCT02767778 clinicaltrial.gov).

Numerous research studies used rat pheochromocytoma PC12 cells to characterize molecular events responsible for PEMFs biological effects (Ivaschuk et al., 1997; Valbonesi et al., 2014). Recently, in these cells PEMF treatment significantly decreased cell death and apoptosis provoked by hypoxia (Vincenzi et al., 2017). Indeed one of the main factors responsible for neuronal cell loss during ischemia is represented by the apoptotic cell death cascade. The apoptotic program in cells may recruit two essential pathways, one extrinsic and the other intrinsic, regulated by death receptors and B-cell lymphoma 2 (Bcl-2) proteins in mitochondria, respectively (Merry and Korsmeyer,

1997). Bcl-2 gene related to cell survival is controlled by the cAMP response element-binding protein (CREB), a nuclear ubiquitous transcription factor necessary to regulate several biologic processes, including cell proliferation and apoptosis, growth, survival and differentiation. Interestingly, neural cells exposed to environmental stress, including ischemic insult, react by inducing cytoprotective responses mediated by heat-shock proteins of 70 kDa (HSP70), brain-derived neurotrophic-factor (BDNF) and mitogen-activated protein kinases (MAPKs). HSP70 is the major inducible member of ubiquitous and highly conserved molecular chaperones HSP family, displaying consistent cytoprotective effects in the nervous system, avoiding proteins malfolding (Amin et al., 1996; Lee et al., 2001; Giffard and Yenari, 2004; Sinn et al., 2007). BDNF exerts a fundamental role in brain repair after ischemia and MAPK activation is recruited following cellular stress (Mehrpouya et al., 2015; Kim 2014; Miloso et al., 2008).

Interestingly, it has been suggested that PEMF treatment induces an external stimulation of cells and tissues, that is followed by complex biological reactions triggered through numerous signaling pathways. As a consequence, it appears important to characterize the molecular mechanisms involved in the protective effects of PEMFs that have not yet been elucidated. Specifically, the pathways involving adenylyl cyclase (AC), phospholipase C (PLC), protein kinase C epsilon (PKC-ε) and delta (PKC-δ), ERK1/2, p38, JNK1/2 mitogen activated protein kinases (MAPK), Akt and caspase-3 have been the focus of this study performed in PC12 neuron-like cells exposed to hypoxia. PC12 cells derive from rat adrenal medulla pheochromocytoma cells and are a useful model to study cerebral ischemia in vitro (Vincenzi et al., 2017). Moreover to better investigate the molecular actors involved in neuronal cell protection induced by PEMFs we have evaluated their regulation of HSP70, CREB, BDNF and the Bcl-2 family proteins in PC12 cells differentiated with NGF exposed to hypoxia.

Materials and Methods

Drugs and materials

4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190) was purchased by Adipogen (Florence, Italy). D-3-Deoxy-2-O-methyl-myo-inositol 1-[(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate] (SH5) and 1,9-Pyrazoloanthrone (SP600125) were from Enzo Life (Florence, Italy). 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) was from Cayman (Florence, Italy). AlphaScreen SureFire p-p38α MAPK (pThr180/Tyr182) assay kit, AlphaLISA SureFire Ultra p-CREB (Ser133) assay kit and ATPlite Luminescence assay sistem were from PerkinElmer (Milan, Italy). PKC-ε translocation inhibitor peptide was purchased by Calbiochem (Milan, Italy). BAD and Bcl-2 ELISA kits were from LSBio (Seattle, North America). Quantikine ELISA BDNF immunoassay was from R&D System (Minneapolis, USA). HSP70 ELISA kit was from Enzo (Milan, Italy). Unless otherwise noted, all other reagents were purchased from Sigma (Milan, Italy).

Cell cultures

Rat pheochromocytoma (PC12) cells were purchased from American Type Culture Collection (Manassas, VA) and were maintained in DMEM F12 medium (Invitrogen, Grand Island, NY) supplemented with 5% FBS (Thermo Scientific, Waltham, MA), 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 500,000/ml and the differentiation was achieved by treatment with 50 ng/ml nerve growth factor (NGF, Sigma, St Louis, MO) (Barbault et al., 2009). Cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS (Thermo Scientific) and the cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 (Reale et al., 2014).

Electromagnetic field exposure system

The neuronal-like cells were exposed to PEMFs generated by a pair of rectangular horizontal coils (14 × 23 cm), each made of 1,400 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 (Varani et al., 2002, 2003, 2008; De Mattei et al., 2009; Ongaro et al., 2012; Fini et al., 2013; Vincenzi 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 × 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.

Hypoxic treatment

Hypoxic exposures were done in a modular incubator chamber and flushed with a gas mixture containing 1% O_2, 5% CO_2 and balance N_2 (MiniGalaxy, RSBiotech, Irvine, Scotland).

Cellular treatments

PC-12 neuronal-like cells were differentiated with 50 ng/ml NGF, then were exposed to 1% hypoxia with and without PEMFs in the presence and in the absence of signalling inhibitors. Inhibitors of kinases, were made up in dimethyl sulfoxide solution (DMSO) and then diluted in cell culture medium (0.1 max 0.2% of DMSO). An equal amount of DMSO was used in control.

ATPlite

ATP is a marker for cell viability because it is present in all metabolically active cells and the concentration declines very rapidly when the cells undergo necrosis or apoptosis. The ATPlite assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. The emitted light is proportional to the ATP concentration within certain limits.

AlphaScreen SureFire p38 MAPK assays

AlphaScreen SureFire p-p38 MAPK (pThr180/Tyr182) assay kit (Perkin Elmer, Milan, Italy) was utilized (Merighi et al., 2017) to detect p-p38 levels. Upon MAPK phosphorylation and excitation at 680 nm, fluorescent signals at 615 nm are emitted. PC12 cells were seeded in 100 µl culture medium into 96-well plates (4,000/well), and incubated at 37°C, in both normoxic and hypoxic conditions, for different time of incubation. After cells stimulation, lysis buffer was added, then donor and acceptor beads linked to specific anti-p-MAPK- and anti-MAPK-antibodies were dispensed, according to manufacturer instructions. Finally, fluorescent signals were detected through an Ensight Perkin Elmer-multimode plate reader (Perkin Elmer, Milan, Italy). Data were normalized to fold of activation above basal pMAPK levels (=100).

HSP70 ELISA

The levels of HSP70 protein present in cell lysates were determined by HSP70 ELISA kit (Enzo, Milan, Italy). In brief, 150,000 PC-12 cells were plated in 6-well dishes and allowed to attach overnight, then changed into fresh medium in the presence of solvent or p38 inhibitor. After 24 h of incubation in normoxic and hypoxic conditions and with or without PEMFs, cells were lysed, and HSP70 protein concentrations were measured by ELISA according to the manufacturer's instructions. Data were normalized to % of activation above basal HSP70 levels (=100). The data are presented as mean ± SE from four independent experiments performed in triplicate.

AlphaLISA SureFire Ultra pCREB assay kit

The levels of p-CREB were determined by the AlphaLISA SureFire p-CREB assay kit (Perkin Elmer, Milan, Italy), according to the manufacturer instructions. PC12 cells were seeded in 100 µl culture medium into 96-well plates, allowed to attach overnight, then the medium was changed into fresh one. After 24 h of incubation in normoxic and hypoxic conditions and with or without PEMFs, cells were lysed and 10 µL of the lysate were transferred to a 384-well Optiplate™ for the assay. After addition of both Acceptor and Donor mix solutions, fluorescent signals were detected through an Ensight Perkin Elmer-multimode plate reader (Perkin Elmer, Milan, Italy). Data were normalized to % of activation above basal pCREB levels (=100).

BDNF ELISA

The levels of BDNF present in cells supernatant were determined by BDNF ELISA kit (R&D System). In details, 150,000 PC-12 cells were seeded in 24 well plate and allowed to attach overnight. The day after cells medium was changed and cells were treated for 24 and 48 h in normoxic and hypoxic conditions and with or without PEMFs. 50 µl of the supernatants were then put in the coated plate supplied by the kit and the levels of BDNF were searched out accordingly to manufacturer instructions. Briefly, after 2 h of incubation, each well was washed and then the total

BDNF coniugated solution was added. After another incubation, wells were washed again and treated with the substrate solution for 30 min. The optical density was determined after the addition of the stop solution, using a microplate reader set to 450 nm.

Bcl-2 and BAD assays

The levels of Bcl-2 and BAD of PC-12 cells were determined by Bcl-2 and BAD ELISA kits (LSBio, Seattle, USA). Briefly, 2,500,000 PC-12 cells were seeded and allowed to attach overnight. The day after the medium was changed and the cells were incubated for 24 and 48 h in normoxic and hypoxic conditions and with or without PEMFs. Cells were then detached and lysed with three cycle of freeze and thaw. After a centrifugation, 100 µl of the supernatants were put in the coated plate supplied by both the Bcl-2 and BAD kits, and incubated at 37°C for 90 min and 1 h, respectively. Then, the 1X biotinylated Detection antibody solution was added. After 1h of incubation each well was washed three times and then the 1X HRP coniugate solution was added. After another washing step, the TMB substrate was added, and the plate was incubated for 15 min in the dark. Finally, the stop solution was added, and the optical density was determined immediately after, using a microplate reader set to 450 nm. Data were normalized to % of activation above basal Bcl-2 and BAD levels (=100).

Statistical analysis

All values in the figures and text are expressed as mean ± standard error (SEM) of independent experiments. Data sets were examined by one-way analysis of variance (ANOVA) and Dunnett's test (when required). A P-value less than 0.05 was considered statistically significant.

Results

Signalling pathways activated by PEMFs to reduce hypoxia-induced cell death

PC12 cells were differentiated with NGF for 1 week and cultured in hypoxia for 48 h to induce cell damage. In our experimental conditions, we found that neuronal-like cells presented a significant reduction of 40% in ATP production, meaning a cell death effect. Interestingly, PEMFs treatment protected cells by reducing cell death to 13%. The role of intracellular signalling triggering this effect as well as the molecules modulated by PEMFs to reduce it were investigated. Specifically, PC12 cells were pretreated for 30 min with 1 μM SQ22,536, U73122, PKC-ε translocation inhibitor peptide and rottlerin as inhibitors of AC, PLC, PKC-ε and PKC-δ, respectively, before incubation with hypoxia for 48 h in serum free medium, in the absence and in the presence of PEMFs. In order to certify the involvement of these signalling pathways in the effect of hypoxia, the comparison between the hypoxia group and hypoxia in the presence of inhibitors group was performed. Statistical analysis
revealed that PKC-ε was involved in the hypoxia-induced cell death whilst PKC-δ signaling contrasted it . In addition, to further assess the involvement of these signalling pathways in the effects of PEMFs against hypoxia, the comparison between hypoxia+PEMFs group and hypoxia+PEMFs+inhibitors group has been measured. The result is that none of these signalling pathways was triggered by PEMFs to exert their protective effects (Fig.1).

Furthermore, to examine whether ERK1/2, p38, JNK1/2 MAPK kinases, Akt and caspase 3 pathways were involved in cell damage-induced by hypoxia, PC12 cells were pretreated for 30 min with 1 μM U0126, SB202190, SP600125, SH5 and 10 μM CASP-3 I, inhibitors of ERK1/2, p38, JNK1/2 MAPK kinases, Akt and caspase 3, respectively, before incubation under hypoxia for 48 h, in the absence and in the presence of PEMFs. The effect of hypoxia on cell vitality was not affected by inhibitors of ERK1/2, p38, Akt and caspase 3, suggesting that these signalling pathways did not

affect cell death induced by hypoxia, while inhibitor of JNK1/2 alone reduced cell death induced by hypoxia. As for the effects of PEMFs, interestingly, SB202190 reverted their protection, suggesting that PEMFs triggered p38 phosphorylation in order to exert their benefical increase in cell survival (Fig.2).

Modulation of p38 MAPK phosphorylation by PEMFs in hypoxia

To confirm the involvement of p38 MAPK kinases in the PEMFs protective effect on cell death induced by hypoxia, a kinetic study on p38 phosphorylation was performed. PC12 cells were treated for different times (0, 10, 30 min) under hypoxia, in the absence and in the presence of PEMFs. As shown in figure 3, PEMFs in hypoxia significantly increased p38 phosphorylation starting from 10 min with a maximum effect at 30 min, thus confirming the involvement of this kinase in the protective signalling pathway activated by them (Fig.3).

Effect of PEMFs on HSP70 levels in hypoxia

To investigate the neuroprotective mechanism of PEMFs, we explored whether the alterations of heat shock protein 70 (HSP70) were involved in hypoxia-induced cell death. To this aim, preliminary experiments were performed on PC12 cells exposed to hypoxia for 2, 4, 8, 16 and 24 h and HSP70 levels were evaluated. HSP70 levels were not altered by hypoxia and hypoxia in the presence of PEMFs until 16 h of treatment. However, we found a slight (114%) stimulatory effect by hypoxia alone after 24 h of incubation in serum free medium. In these conditions (24 h hypoxia) cells exposed to PEMFs showed a significant stimulatory effect on HSP70 (140%) (Fig.4). Interestingly, pretreatment of PC12 cells with the p38 inhibitor SB 1 μM for 30 min abrogated HSP70 increase induced by PEMFs, suggesting that PEMFs trigger p38 to stimulate HSP70 protein to induce neuroprotection (fig.4).

CREB phosphorylation and BDNF regulation by PEMFs in hypoxia

In addition, we evaluated the ability of PEMFs to modulate cAMP response element-binding protein (CREB), a transcription factor downstream HSP70 as well as BDNF, a regulator of neuronal cell survival. Our results show that 24 h of hypoxia alone induced a low increase of CREB phosphorylation, while co-treatment with PEMFs significantly increased it at the same time of incubation (Fig. 5). In addition, at 24 h of hypoxia we found a significant decrease of BDNF levels (40%), but PEMFs co-treatment did not affect them (data not shown). Instead at 48 h of hypoxia the decreased BDNF levels were restored to basal by PEMFs (Fig.5). Pretreatment of PC12 cells with SB 1 μM for 30 min abrogated CREB and BDNF increase induced by PEMFs, suggesting that PEMFs trigger p38 to stimulate CREB phosphorylation and BDNF increase (Fig.5).

Pro-/Anti-apoptotic genes modulated by PEMFs in hypoxia

Finally, we shed light on the modulation exerted by PEMFs on apoptotic genes playing a role in the cell death induced by hypoxia. Specifically, we identified BAD as the principal proapoptotic regulator and Bcl-2 as the most important antiapoptotic one. Figure 6 reports that hypoxia increases BAD after a treatment of 24 h and PEMFs incubation significantly decreased this effect. At 48 h its levels were not more altered by either hypoxia and hypoxia plus PEMFs (data not shown). As for Bcl-2 molecule, we found that hypoxia did not modify its levels following a period of incubation of 24 and 48 h, but PEMFs significantly increased it after 48 h of cotreatment (Fig.6). Pretreatment of PC12 cells with SB 1 μM for 30 min strongly reduced BAD-reduction as well as the Bcl-2 production induced by PEMFs, suggesting that PEMFs trigger p38 to modulate BAD and Bcl-2 in hypoxia (Fig.6).

Discussion

The therapeutical approach with PEMFs exerts several benefical effects in cell biology even if the biophysical, molecular and cellular mechanisms recruited by PEMFs are complex and remain a current area of research interest (Capone et al., 2009; Di Lazzaro et al., 2013). Although significant progress has been reached in the characterization of some pathways that are important for the increase in the knowledge of their effects, a lot of work is still necessary to define all the molecular mechanisms underlying their activity.

In this context the main findings of this study provide for the first time the indications that:

1. PEMFs exposure reduces hypoxic cell death in neuronal like PC12 cells, differentiated with NGF, through p38 MAPK phosphorylation;
2. HSP70, a chaperone molecule exerting strong cytoprotective effects in ischemic brain, is enrolled by PEMFs;
3. CREB and BDNF, important in the regulation of the nervous system and in the protective mechanisms operating after ischemic stroke, are increased by PEMFs;
4. Bcl-2 family of proteins, constituting the intrinsic pathway of apoptosis, is modulated by PEMFs.

In more detail, the results of this work have identified the pathways involved in the protective effects determined by PEMFs against cell death induced by hypoxia, finding a crucial role for p38 MAPK, HSP70 and CREB. Specifically, to test whether PEMFs require p38 activation in order to produce cell protection, PC12 cells were incubated with an inhibitor of p38 and exposed to hypoxia in the absence and in the presence of PEMFs. Interestingly, we found that in this condition PEMFs

lose their capability to protect cells from hypoxia-induced cell death. To confirm the enrollement of p38 by PEMFs, we showed that p38 kinase was rapidly (10-30 min) phosphorylated by PEMFs. Therefore even though this pathway is not the same activated by hypoxia to induce cell death, it is the one triggered by PEMFs to counteract hypoxia-induced cell death. Indeed, a lot of studies report that inhibition of p38 is protective against different type of cell injuries (Kasuya et al., 2018; Lee and Kim, 2017; Cuenda and Sanz-Ezquerro, 2017), but our result reveal that p38 is not involved in cell death induced by hypoxia. Accordingly, other works found a benefical role of p38 in intracerebral haemorrhage, neurodegeneration, apoptosis and oxidative stress (Xu et al., 2015; Back et al., 2015; Yan et al., 2017; Gu et al., 2018). . In addition, further experiments with other selective enzyme inhibitors demonstrate that the protective effects of PEMFs are not mediated by AC, PLC, PKC-ε, PKC-δ, ERK1/2, JNK1/2, Akt and Caspase-3 pathways.

Another molecule investigated in this study as potential modulator of PEMFs effect has been HSP70, a relevant signalling protein which protects the brain from ischemic damage (Rajdev et al., 2000; Tsuchiya et al., 2003; Kacimi and Yenari, 2015). In our experimental conditions, PEMFs significantly increased HSP70 levels and interestingly this effect was abrogated by the p38 inhibitor indicating that PEMFs through p38 activation increase HSP70 molecule to produce beneficial effects against the detrimental injury of hypoxia on cell vitality. Considering that heat shock protein recruitment provides wide cytoprotective effects, there is now great interest in the discovery and development of new agents able to increase it (Kim et al., 2014). Thus, we suggest that PEMFs may be used for this aim, thus acting as potential therapeutic weapon for the treatment of brain ischemia.

The next focus of this work was to evaluate the possible involvement of CREB/BDNF signaling pathway, possessing a critical role in neuroprotection, in the healthful effects modulated by PEMFs in PC12 cells. Interestingly, in our present experiments we found that PEMFs treatment, via p38 engagement, significantly increased CREB and restored the hypoxia-reduced BDNF levels. Accordingly, the same mechanism of neuroprotection mediated by PEMFs was observed following ischemic stroke in mice during the recovery process (Urnukhsaikhan et al., 2017; Li et al., 2014). It

is well known from literature data that the increase of CREB phosphorylation acts on the genome to increase BDNF production playing a crucial role in brain survival and neural cell development (Lonze and Ginty, 2002; Carlezon et al., 2005; Kitagawa 2007; Reus et al., 2011). As a consequence CREB/BDNF pathway activates a protective signalling cascade limiting ischemic cell death (Almeida et al., 2005; Dworkin and Mantamadiotis, 2010; Huang et al., 2015). One of the major factors contributing to neuronal cell death is the cascade of molecular events associated to apoptosis. Indeed cells under stress engage cell survival and cell death intracellular pathways (Haeberlein, 2004; Fricker et al., 2018). Specifically, cell death signaling often affects mitochondria, through processess that are regulated by the pro- and anti-apoptotic Bcl-2 family of proteins (Terranova et al., 2016). Therefore, due to the modulatory effects of CREB in the balance between anti- and pro-apoptotic genes, we thought to shed light in the apoptotic cell death effect induced by hypoxia in PC12 neuronal cells. Our results show that hypoxia increases the proapoptotic BAD protein expression while does not affect the antiapoptotic Bcl-2. Interestingly, PEMFs, through p38 enrollement, counteract BAD enhancement induced by hypoxia and significantly increase Bcl-2 levels, suggesting that the protective effects of PEMFs in hypoxic PC12 cells are mediated by exerting a control on the mitochondrial apoptotic pathway. The same pathway mediated by PEMFs was also demonstrated in the cardioprotective effects aimed to reduce the apoptosis of cardiac cells after myocardial ischaemia/reperfusion injury (Ma et al., 2016). Importantly, our results are in line with neuroprotective mechanisms induced by PEMFs following ischemic stroke provoked in mice through photothrombotic occlusion (Urnukhsaikhan et al., 2017). At present acute ischemic stroke, one of the leading cause of death in the world, is treated with cerebral artery recanalization and antithrombotic therapies (Jauch et al., 2013). However, the short time window of intervention is a limit for clinical success of these approaches (Molina and Alvarez-Sabín, 2009). In this context, the results of this study support the use of PEMFs therapy for neuronal recovery after hypoxic injury thanks to its rapid activation of p38 kinase cascade enrolling

HSP70, CREB, BDNF and finally the antiapoptotic pathway regulated by the Bcl-2 family of proteins (Fig. 7).

In summary, this work may invite further studies on the molecular mechanisms of PEMFs in promoting neuronal cell survival thus contributing to better understanding their therapeutical usefulness in central nervous system injured by hypoxic conditions, as happens during cerebral ischemia.

Conflict of interest statement:

None

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Figures legend

Figure 1- Effect of inhibitors of AC (SQ), PLC (U73122), PKC-ε (PKC-εI), PKC-δ (Rottlerin) at 1 μM on hypoxia-induced cell vitality in neuronal-like PC12 cells NGF-differentiated, in the absence/ presence of PEMFs. *P < 0.01 versus normoxia in the absence (ctr) or in the presence of each inhibitor; #P < 0.01 versus hypoxia in the absence (ctr) or in the presence of each inhibitor; †P<0.01 versus control in hypoxia.

Figure 2- Effect of inhibitors of ERK1/2 (U0), Akt (SH5), JNK1/2 (SP) and p38 (SB) at 1 μM, caspase 3 (CASP-3 I) at 10 μM on hypoxia-induced cell vitality in neuronal-like PC12 cells NGF-differentiated, in the absence/presence of PEMFs. *P < 0.01 versus normoxia in the absence (ctr) or in the presence of each inhibitor; #P < 0.01 versus hypoxia in the absence (ctr) or in the presence of each inhibitor. † P<0.01 versus control in hypoxia; δ P<0.01 versus PEMFs in hypoxia.

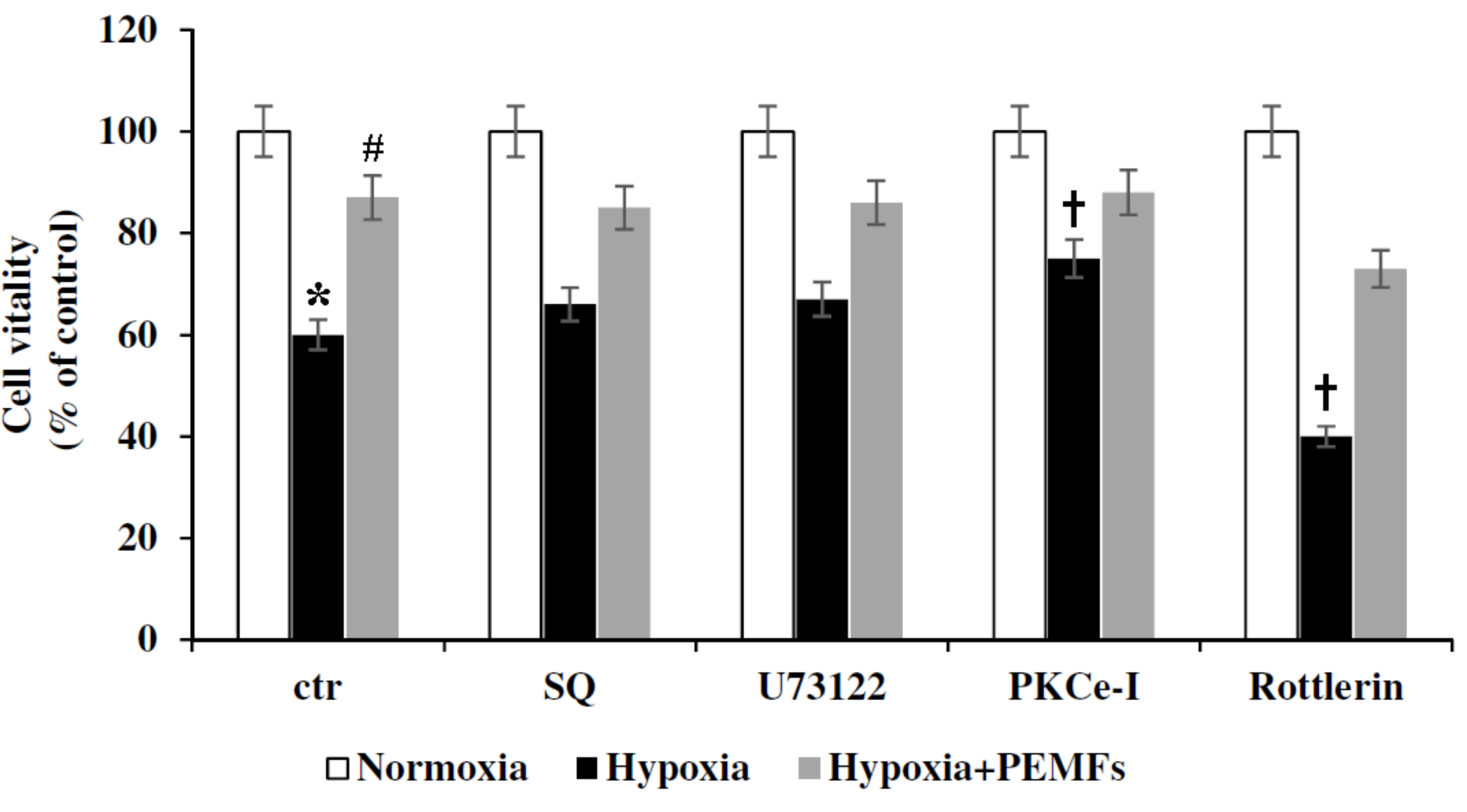
Figure 3- Kinetic effect of PEMFs on p38 MAP kinase activation at 0, 10, 30 min in neuronal-like PC12 cells NGF-differentiated in hypoxia. *P < 0.01 versus hypoxia; #P < 0.01 versus hypoxia at each time point.

Figure 4- Effect of p38 inhibitor (SB 1 μM) on HSP70 levels in neuronal-like PC12 cells NGF-differentiated in normoxia and hypoxia, in the absence/presence of PEMFs. *P < 0.01 versus control in hypoxia (ctr). *P < 0.01 versus hypoxia.

Figure 5- Effect of PEMFs on CREB phosphorylation (24h) and BDNF modulation (48h) in normoxia and hypoxia, in the absence and in the presence of the p38 inhibitor (SB 1 μM), in neuronal-like PC12 cells NGF-differentiated. *P < 0.01 versus normoxia; #P < 0.01 versus hypoxia.

Figure 6- Effect of PEMFs on BAD and Bcl-2 (24 and 48h) modulation in normoxia and hypoxia, in the absence and in the presence of the p38 inhibitor (SB 1 μM), in neuronal-like PC12 cells NGF-differentiated. *P < 0.01 versus normoxia at each time; #P < 0.01 versus hypoxia at each time.

Figure 7- Schematic representation of PEMFs induced signalling in neuronal-like PC12 cells NGF-differentiated to relief hypoxia-induced cell death. A rapid activation of p38 kinase cascade followed by HSP70, CREB, BDNF recruitment and finally the antiapoptotic pathway regulation is shown.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Cell vitality
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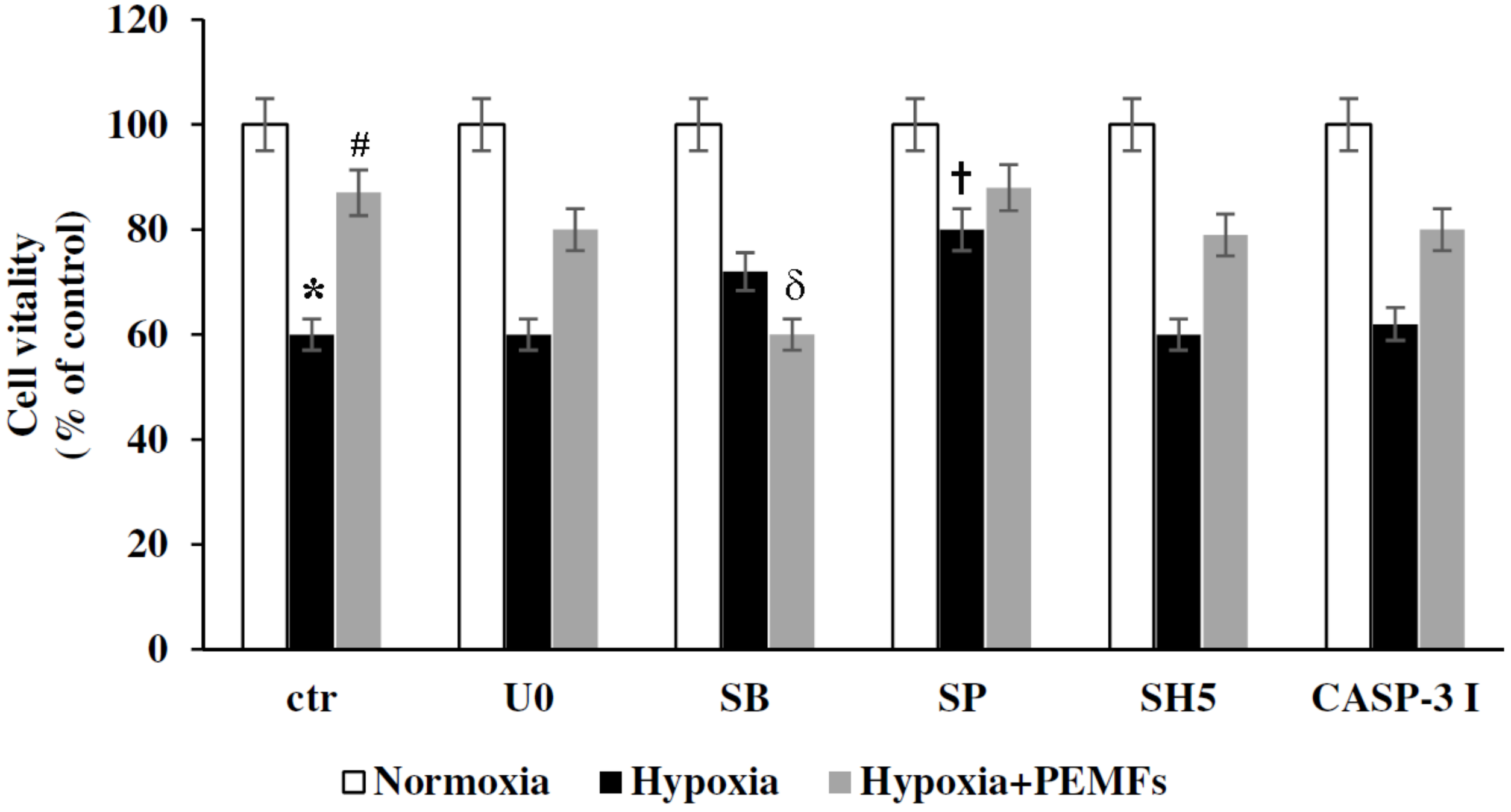
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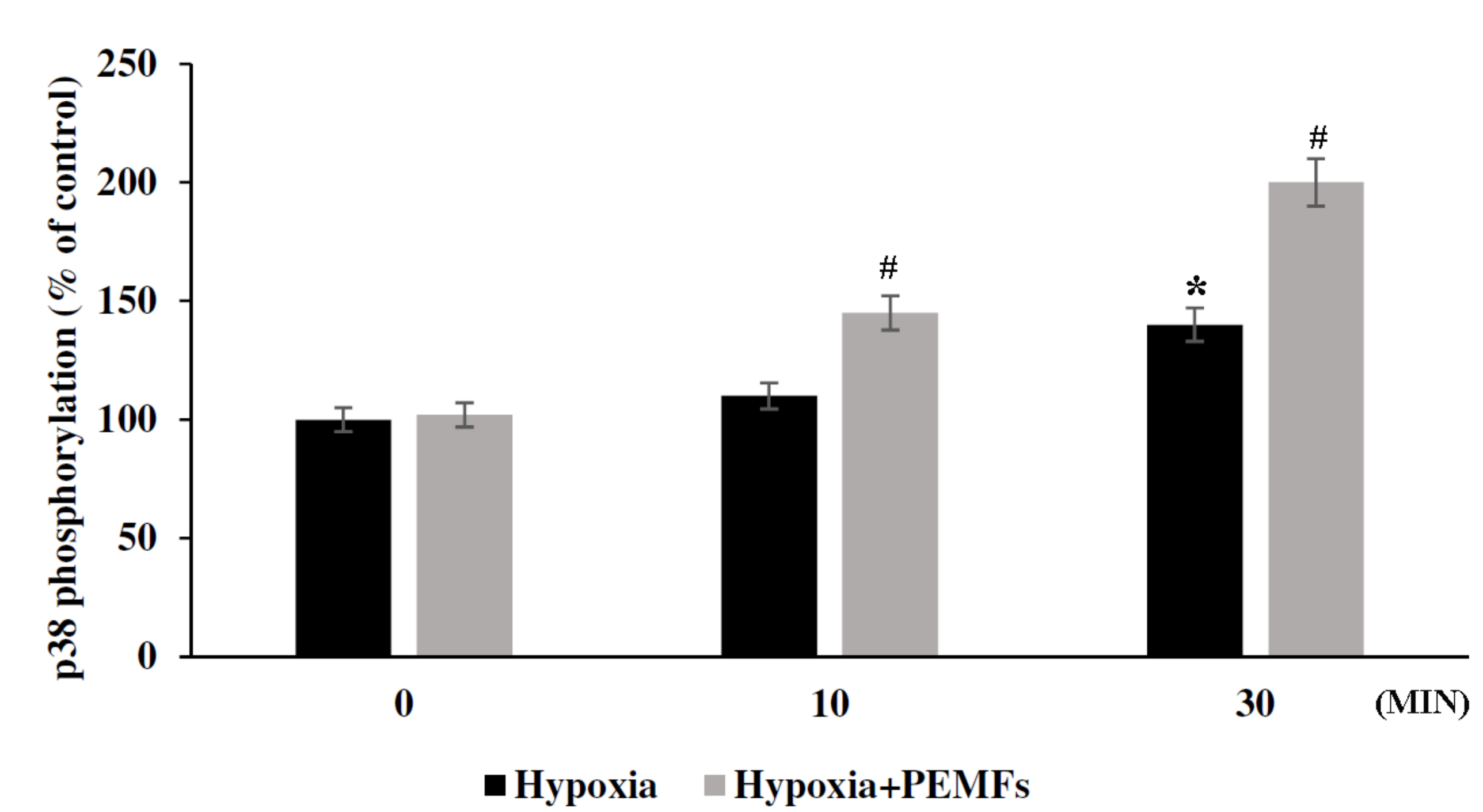
Normoxia
Hypoxia
Hypoxia+PEMFs


p38 phosphorylation (% of control)
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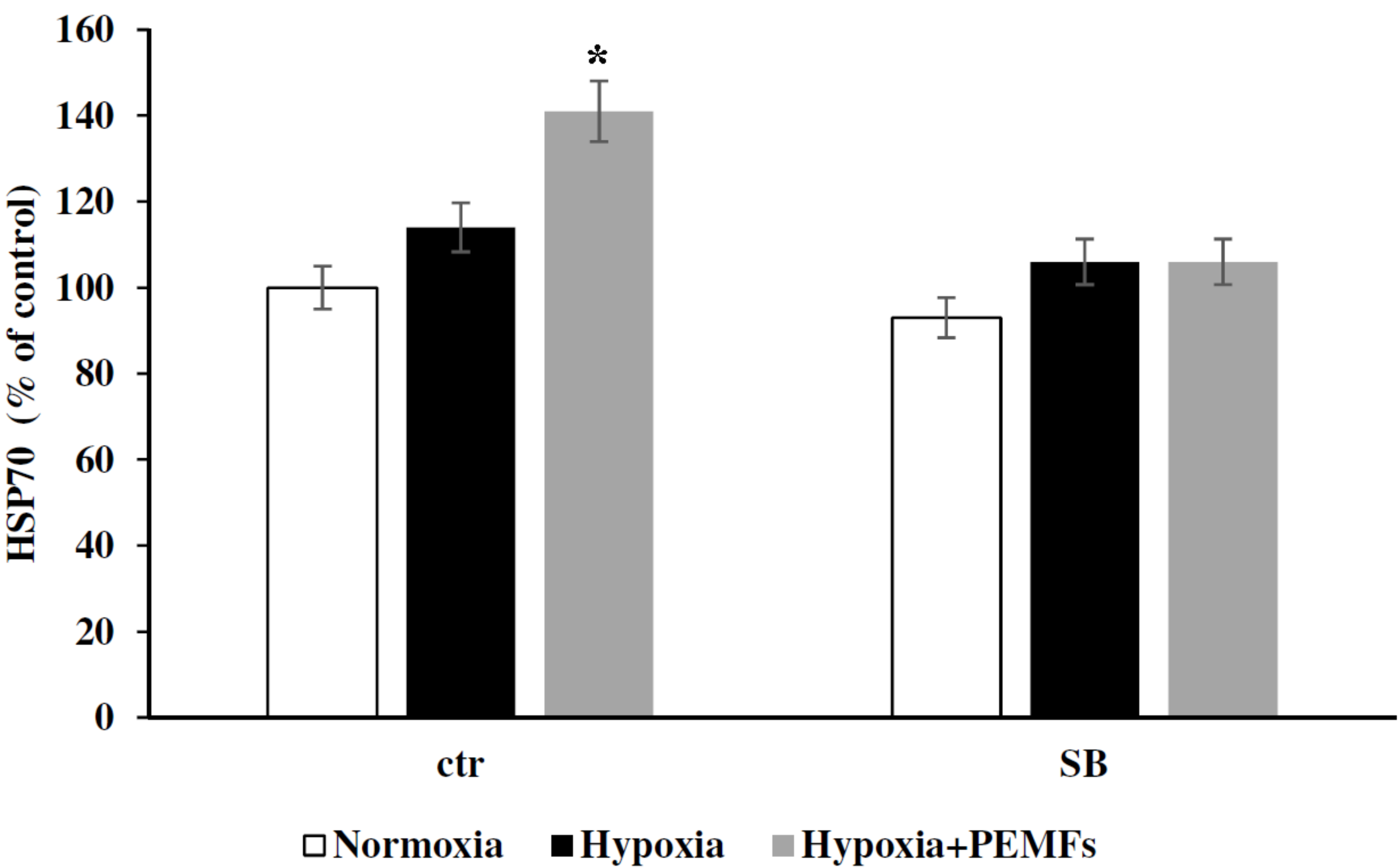
Hypoxia
Hypoxia+PEMFs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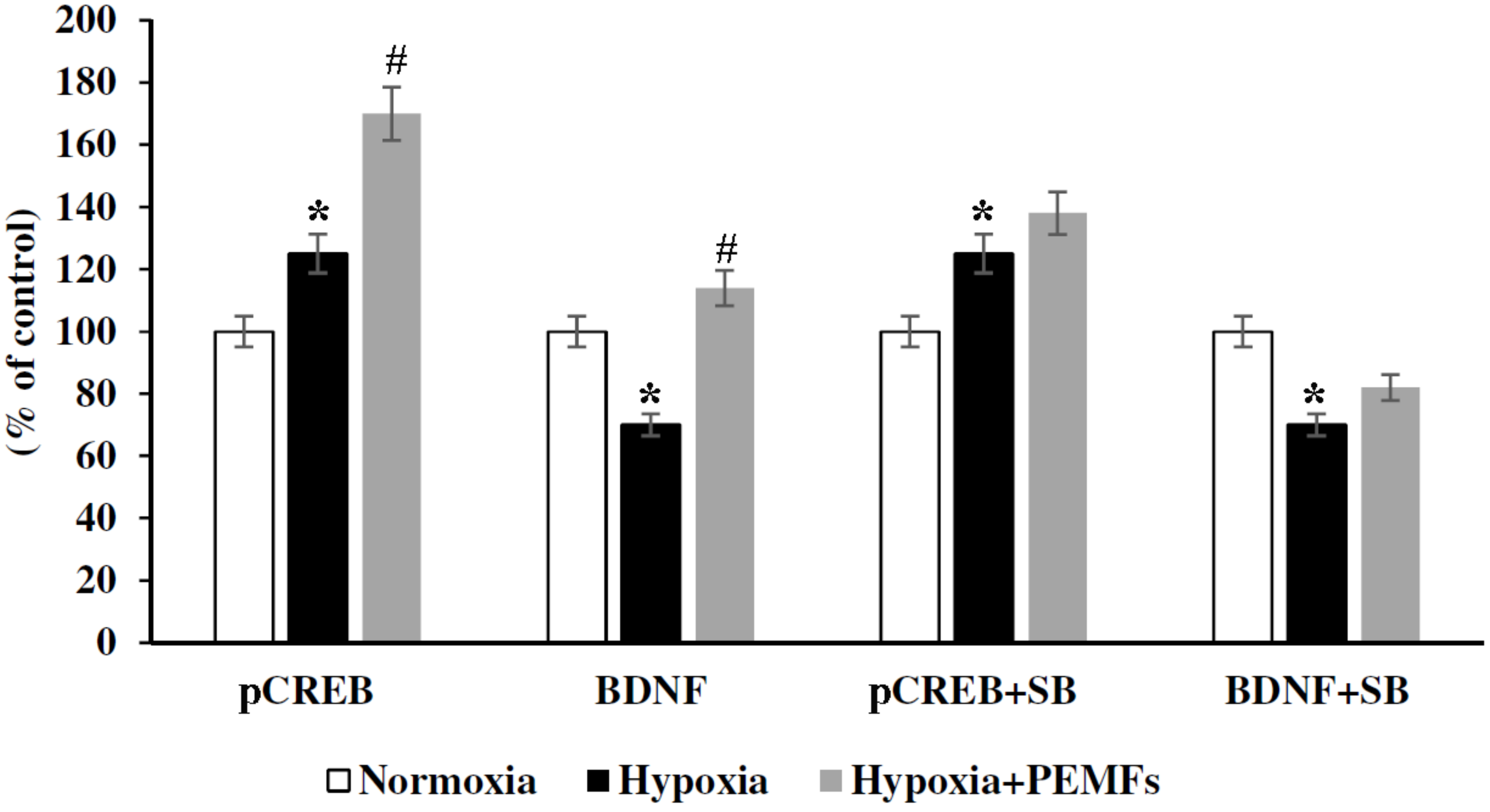
Hypoxia
Hypoxia+PEMFs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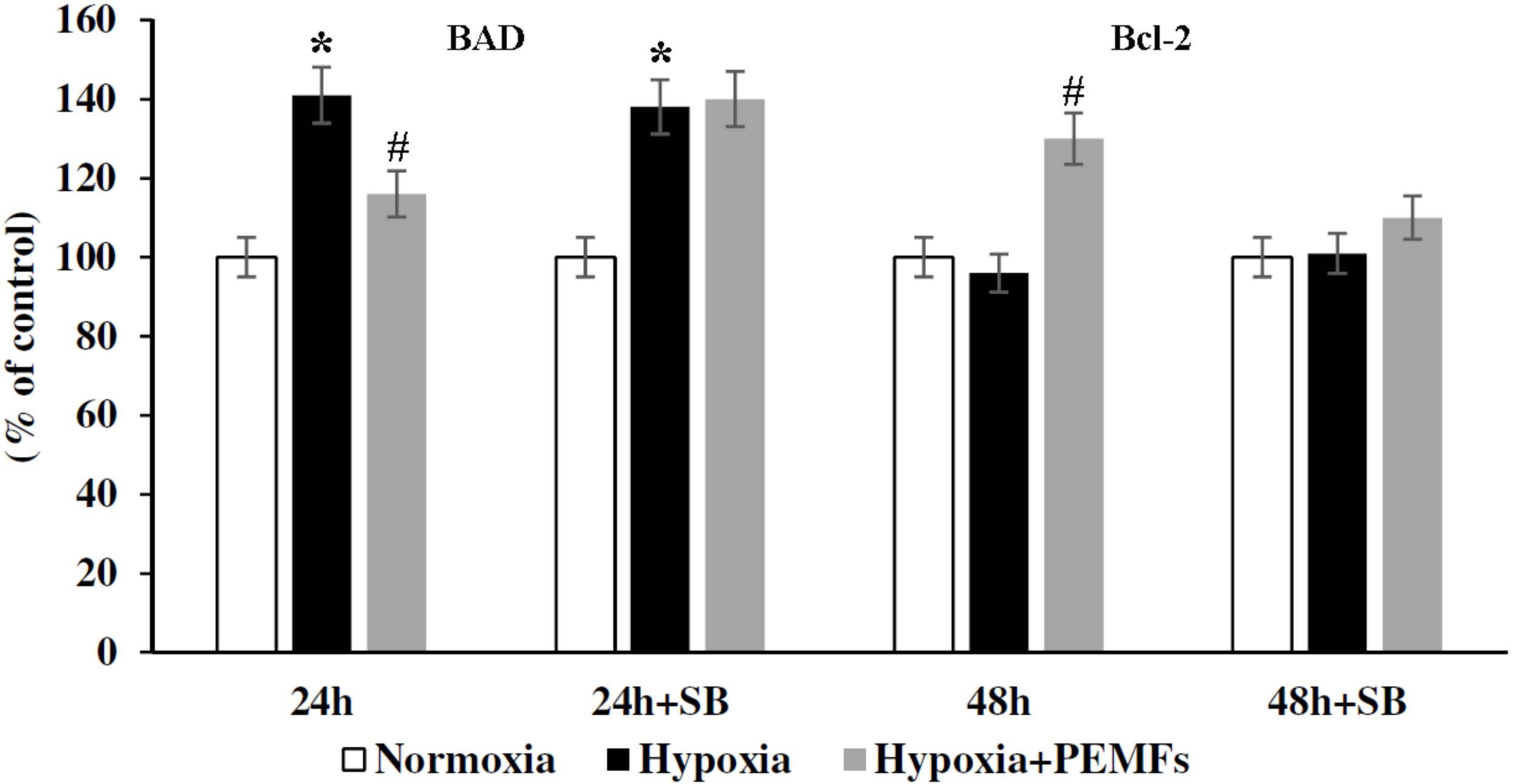
pCREB+SB
BDNF+SB
Normoxia
Hypoxia
Hypoxia+PEMFs


BAD
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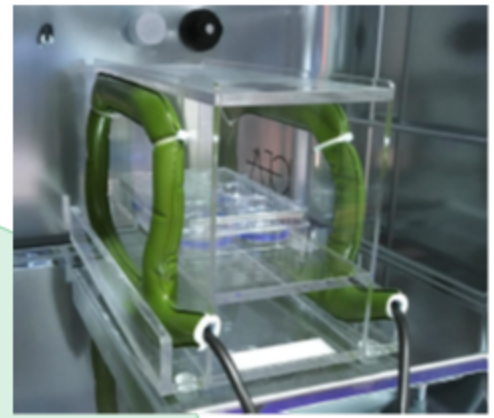
24h
24h+SB
48h
48h+SB
Normoxia
Hypoxia
Hypoxia+PEMFs

HYPOXIA



PEMFs

30 min — p38 ↑

24 h — HSP70 ↑

24 h — pCREB ↑

48 h — BDNF ↑

Bcl-2 ↑ BAD ↓