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PEMFs and cerebral ischemia: the pathways behind their beneficial effects

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INTRODUCTION

ISCHEMIA

According to the World Health Organisation, over 15 million people worldwide, every year, that is one in 400 people, suffer a stroke. People aged 65 and over are the most affected, however all ages are at risk, including younger people, children and infants (Ezzelarab et al., 2020). Stroke is one of the world's biggest killer, responsible of almost 6 million of deaths and second just to heart disease. It is the most frequent cause of long-term disability and 40% of the affected patients do not regain independence (Allen and Bayraktutan, 2009).

Strokes can be distinguished in ischemic or haemorrhagic, with a prevalence of 85% of the cases belonging to the first group (Jayaraj et al., 2019).

Ischaemic stroke results from the interruption or the acute reduction of blood flow, and so of the oxygen level, in cerebral arteries. In details, two different areas can be distinguished: the core, where there is an instant neural death, and a surrounding area, called penumbra, moderately hypo-perfused, that keeps structural integrity but with a decrease or loss of function (Phan et al., 2002).

A decreased concentration of oxygen able to comprise cells' capacity to sustain life is defined hypoxia. With the term ischemia, however, although the terms are often interchanged, is defined the scarcity of oxygen and of nutrients, due to a poor blood supply to the tissue (Li et al., 2017).

This event is caused by an arterial occlusion, due to embolus or thrombus (Catanese et al., 2017). Depending on the artery occluded, different functional and metabolic irregularities occur and so the dimension of the ischemic area in the brain involved may vary (Dague et al., 2017).

The episode is quite deleterious for the brain, that needs a constant supply of oxygen and glucose to fulfil its functions and to maintain the viability of its cells. Indeed, the entire central nervous system (CNS) consumes a high level of oxygen, making it very sensitive to a change in this molecule concentration (Luo et al., 2011).

Because of this, the cells belonging to the core, characterized by <20% of baseline blood flow levels, having ATP stores depleted and the energy metabolism irreversibly failured,

are irreversibly damaged, while the one located in the ischemic penumbra may still be saved.

The cascade triggered by the lack of oxygen starts soon after the event and has been extensively examined. In particular, the major factors found to be involved seems to be excitotoxicity, that indicates the neuronal damage resulting from excessive activity of excitatory amino acids (EAAs) in ischemic brain, and calcium overload (Fig.1) (Chen et al., 2011).

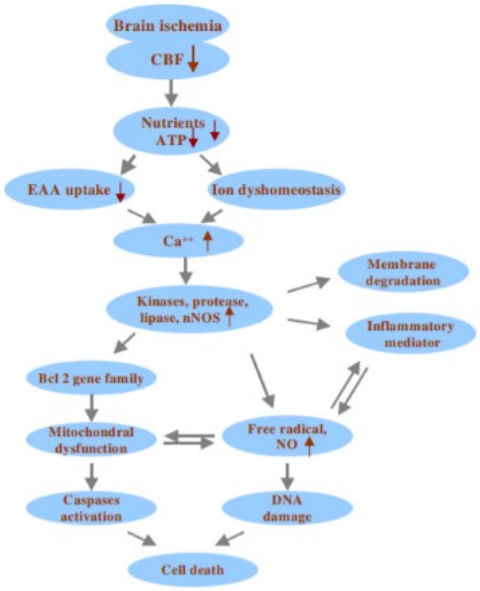

Figure 1. Schematic overview of selected cellular events in the ischemic brain. The ischemic event begins with reduced blood flow to the area supplied by the occluded arteries. The lack of oxygen, glucose, and other nutrients leads to an ischemic cascade culminating in cell death. EAA = excitatory amino acid; ATP = adenosine triphosphate; CBF = cerebral blood flow; nNOS = neuronal nitric oxide synthase; NO = nitric oxide; ↑ and ↓ denote increase and decrease, respectively (Chen et al., 2011).

A central role is played by glutamate: due to the mitochondrial failure in ATP-synthesis and the reversal of the glutamate uptake transporter, its level increases within the first minutes (Choi and Rothman, 1990). Its overload leads to the overactivation of the AMPA and NMDA ionotropic receptor subtypes, causing an increased influx of calcium, sodium and water into neurons. The calcium influx activates a series of catabolic processes, stimulating proteases, lipases and nucleases (Ankarcrona et al., 1995), but also nNOS, PLA2 and other Ca2+ dependent enzymes that lead to the production of reactive oxygen species (ROS), finally inducing cell death (Dirnagl et al., 1999; Lo et al., 2003).

To contrast this, in order to improve the outcome in stroke patients, different clinical trials that targeted NMDA and AMPA receptors have been tried, unfortunately with no success (Ginsberg, 2009). In fact, this pathway is not the only one involved in this deleterious process.

Other studies have brought to light the existence of a glutamate-independent calcium influx that follows oxygen-glucose deprivation and have reported the failure of glutamate receptor blockade in stopping cell death after a very intense oxygen-glucose deprivation (Aarts et al., 2003). In addition, the non-neuronal cell types engaged in the ischemic area, such as astrocytes, vascular and microglial cells, were not considered.

In excitotoxicity, an important role is performed also by NO and the related oxidative products, that are involved in the inhibition of key mitochondrial enzymes, in the mitochondrial transition pore formation, DNA damage, PARP and Ca2+ permeable TRPM7 channels (Pacher et al., 2007). In addition, NO is able to modify protein groups, altering the targets' functions; it can modify caspases, metalloproteases (Gu et al., 2002), and the glycolytic enzyme GAPDH (Nakamura and Lipton, 2009), regulating cell survival.

Another process evoked by ischemia, that plays a major role in this condition, is neuroinflammation, that begins within a few hours of onset and typifies the secondary or delayed response to ischemia. This response requires the activation of microglia, astrocytes and hematogenous cells, that are recruited by cytokines, adhesion molecules and chemokines secreted by the dying and dead cell.
Among these, lipopeptides, advanced glycation end-products (AGE), modified lipids, heat shock proteins, hyaluronic acid, and the nuclear protein HMGB1 are known to activate the innate immune system by engaging toll like receptors (TLRs). These receptors are expressed in multiple cells and act as a detector for "danger signals", triggering NFκb-dependent inflammatory signaling (Oppenheim and Yang, 2005; Wang et al., 2007).

Once stimulated, cells communicate with each other both in a paracrine and autocrine way, activating all the cells of the adjacent area. In fact, neutrophils, monocytes and T cell subsets have been implicated as modulators in the infarct progression (Bennet and Bennet, 2020).

In particular, a key role is played by microglial cells, the resident macrophages of the brain, that are activated soon after the ischemic event. These cells are highly dynamic: exploring the surrounding environment with their processes, they keep the homeostasis of the system. When activated, they react in different ways, including migrating toward the site of injury, phagocyting the debris (Hickman et al., 2018; Hammond et al., 2018) and releasing numerous pro-inflammatory mediators, such as interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α), that aggravate brain damage (Jayaraj et al., 2018).

At least early on, inflammation is known to amplify the ischemic lesion; on the contrary, in the late post-ischemic period, it improves tissue repair. However, the therapeutic trials that targeted the inflammatory response failed to show clinical benefit (Sughrue et al., 2004), underlying the difficulty in controlling such a complex environment.

The derived damage affects the blood brain barrier (BBB) too, whose principal function is to regulate the cerebral homeostasis. The BBB is formed by endothelial cells, that connect through adherent and tight junctions and lack fenestration like nowhere else in the body, a basement membrane, pericytes and the end feet of astrocytes. Pericytes, through their contractile elements, regulate the vascular perfusion pressure and capillary blood flow surrounding the barrier (Fisher, 2009), while astrocytes feet control cerebral capillary blood flow (Wolburg et al., 2009). Finally, the basal lamina, constituted of extracellular matrix proteins, interacts with endothelial cells through integrin molecules.

It is known that ROS have the endothelium and the vascular system as target, affecting the permeability of the BBB. In particular, it has been reported that the reperfusion-injury is able to activate matrix metalloprotease and so to provoke, on BBB, the degradation of the endothelial cells that start to express stress fiber, to contract, due to modification on tight junctions, to disassemble and to modify the lipid oxidation, leading to an oxidative neurovascular unit damage (Kahles and Brandes, 2012).

In addition, excitotoxicity is known to affect astrocytes, leading to both the inhibition of their repair and their feet disruption, and causing the demolition of the BBB (Chen et al., 1999).

Unfortunately, the harmful effects are not only found at molecular and cellular levels; in fact, the disruption of the BBB, and so the augmented vascular permeability, leads to an edema that causes an inadequate perfusion of the affected tissue bed (Bektas et al., 2010).

The final outcome is clinically negative: it has been demonstrated that the degree of disruption of the BBB correlates with the severity of patient outcomes.

The vasodilatation induced by ROS has been demonstrated to exacerbate neuronal damages too, transporting pro-inflammatory mediators, increasing the local neuroinflammation and causing hyperemia.

Because of this, the subsequent restoration of blood flow in the damaged area, known as reperfusion, not only provides oxygen again, but is often associated to an increased injury of the affected tissue (Chen et al., 2010; Eltzschig and Eckle, 2011), carrying both pro-inflammatory cytokines and mediators and cells of the immune responses to the site.

Subsequent to ischemia and reperfusion and due to the neuroinflammation and the continuous damage mediated by free radical oxides, the most known consequence is hemorragia (Wang and Lo, 2003).

To date, the only available treatments for ischemic stroke patients are the antithrombotic therapies and the cerebral artery recanalization (Jauch et al., 2013). However, the treatments are not always effective, and could lead to complications. In fact, the vessel recanalization outcome could be successful and save the tissue, could lead to no-reflow in the ischemic tissue bed, or results as deleterious, causing cell death or hemorrhagic transformation.

PULSED ELECTROMAGNETIC FIELDS (PEMFs)

Electromagnetic fields (EF) are found naturally in our environment; however, their biological effects are still poorly investigated. In detail, the human population is exposed to two types of EF: the radio frequency magnetic fields, associated to the use of phones and of all the wireless devices, and the extremely low frequency, produced by electrical and electronic appliances (Capone, 2009).

The capability of the second type to produce a positive outcome in different systems of the body has been described by several studies.

A prolonged exposure is reported to improve social recognition memory (Vazquez-Garcia et al., 2004), spatial learning (Liu et al. 2008) and human standing balance (Thomas et

al., 2001). Low frequency EF also appeared to influence the sensory system, changing the pain threshold in both animals and humans.

In addition, their influence in brain activity is also supported by some neurophysiological studies that indicate changes in electroencephalographic activity with an increase in alpha rhythm (Bell et al., 1992, 1994; Cook et al. 2004; Lyskov et al. 1993). However, the significance of these data in a clinical and behavioural way is still not clear. In vitro studies conducted in neuron-like cells showed that Low frequency EF alters gene-expression (Campbell-Beachler et al. 1998; Pirozzoli et al. 2003), enhances neurite outgrowth (Blackman et al. 1993; McFarlane et al. 2000), decreases apoptosis (Oda and Koike 2004) and, through the upregulation of the Calcium-channel, facilitates neuronal differentiation of neural stem/progenitor cells (Piacentini et al. 2008). Alteration of the Calcium signaling, in rat hippocampus, were shown to modify NMDA receptor functions when exposed to 50 Hz magnetic field.

However, one concern emerged from these studies was to address the electromagnetic environmental pollution, considering that the waveform of the signal is sinusoidal, and the peak values of the magnetic field are in the range of microTesla. Thus, the change in pulsed electromagnetic fields (PEMFs) that are still generated by pulsed signals, but with a peak value of the magnetic field in the range of milliTesla (mT) and a fast rate of change of the magnetic field (dU/dt) (milliseconds) (Capone et al., 2009).

PEMF signals are short bursts of electrical current, generated by quite simple devices, allowing a non-invasive (external) application to injured tissue, without interfering with nerve or muscle functions. Different exposure systems with these characteristics have been developed and utilised to explore biological effects and their therapeutic applications for a wide range of clinical conditions (Pena-Philippides et al., 2014).

- PEMFs IN BONE REPAIR

Numerous in vitro or in vivo experiments reported the ability of PEMFs to influence different physiologic systems. Therefore, the aim of many studies was to analise the biophysical stimulation induced by PEMFs, to use them as potential alternative to the pharmacological treatments for the related pathologies (Varani et al., 2017).

In particular, Bassetti and colleagues were the first to use PEMFs treatment for animal bone repair, paving the way for its use as a therapy for human nonunion that led to the FDA approval in 1979 (Bassett et al., 1974). To date, different studies reported its beneficial effects on healing of delayed union and nonunion of bone fracture. To mention, the study of Goldberg and colleagues, that examined 11,000 cases of consolidation failure patients treated with PEMFs, finding a healing rate of 75% (Goldberg et al., 1982), and the examination of the European PEMFs treatment of bone nonunion, that found a statistically significant difference with the dummy group (Qui et al., 2020).

Studies on PEMFs exposure discovered their ability in modulating cartilage and bone metabolism, promoting both chondrocyte and/or osteoblast proliferation and the production of the extracellular matrix components (Chalidis et al., 2011). These effects are shown to be beneficial in the treatment of fractures, boosting their healing (De Mattei et al., 1999; Lin and Lin, 2011). In fact, different studies showed that PEMFs can induce osteoblastogenesis, the differentiation of osteoblasts (Sollazzo et al., 2010) and proteoglycan synthesis (Vincenzi et al., 2013).

In addition, PEMFs treatment was reported to increase osteoblast activity while reducing osteoclast formation (Funk, 2018). Zhai et al. demonstrated that the use of PEMFs for 2 hours/day ameliorates the cytoskeletal organization, upregulates the expressions of gene proliferation-related and increases the production of the protein collagen type 1 of the Runt-related transcription factor 2 and of Wnt/β-catenin signaling (Zhai et al., 2016). In guinea pigs, PEMFs treatment (75 Hz) reduced all symptoms of knee osteoarthritis (Veronesi et al., 2014) and in animal models it showed to have chondroprotective effects on joint cartilage (Funk, 2018).

In fact, the ability of PEMFs to stimulate the proteoglycan synthesis without affecting the degradation was reported, indicating their possible use to maintain the function and the integrity of the cartilage (De Mattei et al., 2003). Interestingly, it was observed that the combination of bone marrow concentrates and PEMFs enhanced the osteochondral regeneration, improving the cartilage cellularity and the matrix parameters (Veronesi et al., 2015).

In addition, PEMFs treatment was able to reduce the production of some of the most relevant pro-inflammatory cytokines in human synoviocytes, chondrocytes, and osteoblasts (Varani et al., 2017), suggesting that healing may be mediated by decreasing the inflammation, too.

An important process involved in healing is angiogenesis. It was demonstrated that PEMFs can increase the production of growth factor beta-2 (Tepper et al., 2004), collagen I, anti-inflammatory prostaglandins and the Vascular Endothelial Growth Factor (VEGF)-A-mRNA transcription by fibroblasts (De Girolamo et al., 2013), indicating an inclination towards proliferation and vascular density. The increase in the proliferation rate was proved in different cell lines: murine osteosarcoma (Miyagi et al., 2000), chondrocytes and osteoblasts (Lohman et al., 2005). In the last one, PEMFs treatment was able to augment the synthesis of nitric oxide (NO). This mediator is known for its vasodilatory effects, but it exerts different functions on the vascular wall such as contrasting the apoptosis (Dimmeler and Zeiher, 1999), controlling cell migration and angiogenesis (Murohara et al., 1998) and decreasing the inflammatory response (Spiecker et al., 1997). Recent experiments reported that PEMFs can decrease the interleukin IL-1β levels, while increasing the expression of the Transforming Growth Factor (TGF-β) family members.

Different clinical studies on arthroscopic surgery reported a faster function recovery after the surgery in PEMFs-treated patients than in the control group (Vincenzi et al., 2013; Varani et al., 2017), and that benefits were maintained at the three years follow-up. The analysis of randomized controlled trials emphasizes that PEMFs treatment significantly reduces the radiological and healing time of acute fractures (Varani et al., 2017).

- PEMFs AND PAIN RELIEF

The PEMFs exposure has been reported to produce analgesic (antinociceptive) effects in different organisms. In fact, the effects of PEMFs on pain has already been described in rats, mice, snails, pigeons and humans (Shupak et al., 2003, 2004; Prato et al., 2000).

PEMFs treatment was used in a randomized, double-blind, sham-controlled clinical trial, in patients with chronic generalized pain affected by fibromyalgia (FM). The exposure was performed with a device containing coils positioned bilaterally over the cranium, and after a twice daily 40 min exposition for 7 days, a significant result was reported. Recently, an increase in the thermal pain thresholds among human volunteers exposed to PEMFs has been reported (Shupak et al., 2004).

This kind of treatment could be the answer when patients suffer from intolerance to chronic and high doses of e.g. nonsteroidal anti-rheumatic drugs. Thanks to pain reduction, an improvement in mobility and in the ability in daily activities was reported,

with benefit for both patients' passive physical movement and physical training. Clinical studies reported indeed the beneficial effect of PEMFs also as treatment for arthritis and neuropathy (Funk, 2018).
PEMFs treatment was also described as beneficial for osteoarthritis and for the related pain, stiffness and physical function in elderly patients. A clinical study in the elderly described a significant improvement in the PEMFs treated group compared to the control group after three months (Iannitti et al., 2013).

Randomized, double-blind, sham-controlled pilot trials in postoperative pain relief following breast surgery and a single-arm, open-label pilot study of failed back surgery syndrome pain showed the efficacy of PEMFs treatment. The analysis of PEMFs therapy in treating postoperative pain and edema was also found to be effective (Kubat et al., 2015).

Moreover, besides the reduction of the postoperative pain, PEMF therapy significantly reduced the narcotic use in the immediate postoperative period (Varani et al., 2017).

The beneficial effect of PEMFs on pain seems to be related to the reduction of inflammation (Kubat et al., 2015), however, little is known about the mechanisms involved. In vivo and in vitro studies seem to indicate the involvement of PEMFs in the modulation of gene expression related to ion channels activation. In fact, in rodents, PEMFs were able to activate Na+ and Ca2+ voltage-gated ion channels, which are known for triggering pathways finally involved in the gene expression modulation. In vitro studies also reported a change in the profile of different inflammation and analgesia associated factors (Moffett et al., 2011); among these: the increase in opioid precursor expression (Moffett et al., 2012). In addition, in postoperative wound exudates of PEMF-treated tissue a decreased level of IL-1β peptide if compared to the sham-treated ones, and a corresponding lower pain scores (Rohde et al., 2010) was found, indicating a correspondence between the anti-inflammatory role of PEMFs and pain reduction.

- ANTI-INFLAMMATORY EFFECTS OF PEMFs ON STROKE

PEMFs treatment has shown beneficial effects also in animal model of diseases of the CNS such as ischemia, Alzheimer's and Parkinson's diseases (Grant et al., 1994; Arendash et al., 2010; Wang et al., 2010). It was reported, specifically, its ability to promote angiogenesis and to reduce infarct size and neuroinflammation (NI) through the decrease

of pro-apoptotic mediators and the increase of pro-survival molecules (Urnukhsaikhan et al., 2017).

PEMFs treatment has been reported to possess anti-inflammatory and anti-oxidative properties. In nucleus pulposus cells it decreased the production of IL-1β and TNF-α (Zou et al., 2017), while in osteoblasts it reduced ROS levels, enhancing the antioxidative stress response (Ehnert et al., 2017).

Neuroprotective effects were showed in the NS. In spinal cord injury (SCI), PEMFs were able to decrease the following iron-induced tissue damage, to improve the recovery of motor function in SCI rats and to decrease the inflammation and oxidative stress in the damaged spinal cord, through a mechanism that is still not clear, but that might involve the activation of HSP70 (Wang et al., 2019).

In in vitro model of AD, PEMFs could modulate the expression of microRNAs, stimulate tissue regeneration (Capelli et al., 2017) and enhance visual memory, while in PD, they significantly improved the typical motor symptoms (Pena-Philippides et al., 2014).

PEMFs treatment was discovered as beneficial also in hypoxia related conditions. In human renal proximal tubular cells, PEMFs decreased the cells induced death by diminishing the ROS production (Varani et al., 2017).

In ischemic insult, PEMFs application prior to, during and after, protected the heart against ischemia/reperfusion-induced cardiac contractile dysfunction and heart injury (Bialy et al., 2015). In rats, PEMFs limited the area of necrosis caused by acute experimental myocardial infarcts. *In vivo* studies reported a faster healing of tissue damaged by ischemia when treated with PEMFs (Grant et al., 1994).

PEMFs treatment had beneficial effects also in the CNS: in a distal middle cerebral artery occlusion in mice, it showed to modify the expression profile of pro- and anti-inflammatory factors in the hemisphere ipsilateral to ischemic damage and to decrease the infarct size (Pena-Philippides et al., 2014). The infarct size dimension was reduced also in rabbits after transient focal ischemia (Grant et al., 1994).

There are still few studies that characterise the mediators involved in this protective effect; however, it was demonstrated that, in ischemic stroke, PEMFs activate the brain derived neurotrophic factor/tropomyosin receptor kinase B/protein kinase B signaling pathway (Wang et al., 2019).

In healthy subjects PEMFs exposure modulated neuronal activity through the increase of cortical excitability (Capone et al., 2009). The safety and the tolerability of PEMFs treatment in patients with acute ischemic stroke was investigated and demonstrated in an open-label, one arm, dose-escalation, exploratory study (Capone et al., 2017). These results led to a multicentric, prospective, randomized, placebo-controlled, double-blind study whose aim was to evaluate PEMFs as a therapy in acute ischemic stroke (NCT02767778 clinicaltrial.gov).

AIM OF THE STUDY

Stroke is a major public health problem; one of the world biggest killer and a leading cause of serious, long-term disability in the survivors (Pena-Philippides et al., 2014).

Human brain is a highly metabolical active organ, very sensitive to oxygen and nutrients concentration. The interruption or the acute reduction of blood flow is accompanied by different processes such as bioenergetic failure, excitotoxicity, oxidative stress and ROS production, blood–brain barrier dysfunction, microvascular injury and postischemic inflammation, that damage the ischemic region resulting in the apoptotic death of endothelial cells, neurons, and glia (Brouns and De Deyn, 2009).

Soon after the stroke, the inflammatory response is triggered in the damaged environment, with release of inflammatory cytokines, such as IL-1α and -1β, IL-6, IL-10, and TNF-α, the activation of microglia and astrocytes, and the infiltration of monocytes into the ischemic area (Lucas et al., 2006; Perera et al., 2006). The pro-inflammatory response is protracted in time; in fact, it has been described up to several weeks after stroke.

Neuroinflammation has been reported to be a critical key in the clinical outcome: greater is the level of anti-inflammatory cytokines, lower is the infarct size and better the outcome. In fact, anti-inflammatory processes and cytokines are necessary for tissue healing and the restoration of the normal homeostasis (Perera et al., 2006; Lakhan et al., 2009).

Different trials tested numerous drugs that targeted several mediators involved in inflammation, however, to date, the only therapeutic approaches are the antithrombotic therapies, associated with time limitations (3-h therapeutic window), and the cerebral artery recanalization (Jauch et al., 2013). In addition, both cases present a risk of complications, such as hemorrhage and the reperfusion/ischemic injury (Lakhan et al., 2009).

The use of PEMFs, a non-invasive treatment based on pulsed electromagnetic fields, that are characterised by a repetitive variation in the intensity of the magnetic field over time, with a waveform that gets up and down rapidly, may represents a possible and valid option. Indeed, FDA-approved PEMFs, as adjunctive therapy, have already been utilised in different conditions, with a positive outcome and without producing heat or interfering

with nerve or muscle function (Pilla et al., 2013). The use of this relatively simple device has been reported as positive also in ischemic rabbits, in which a reduction of infarct size was described (Grant et al., 1994).

PEMFs treatment seems also to modulate inflammation in different conditions; in particular, it has been shown to decrease the cytokine IL-1β after traumatic brain injury in rats (Rasouli et al., 2012) and after breast reduction (Rohde et al., 2010), and the pain following breast augmentation (Heden and Pilla, 2008) and osteoarthritis (Nelson et al., 2013).

Positive effects have been demonstrated also on visual memory and on motor symptoms in Alzheimer's and Parkinson's patients, respectively (Sandyk, 1994; 1996).

An open-label, one arm, dose-escalation, exploratory study demonstrated the safety and tolerability of PEMFs in patients affected by acute ischemic stroke (Capone et al., 2017). Therefore, a multicentric, prospective, randomized, placebo-controlled, double-blind study to evaluate the effectiveness of low-frequency pulsed electromagnetic fields in acute ischemic stroke, as non-invasive and safe tool to promote recovery, has been initiated (NCT02767778 clinicaltrial.gov).

Even though the beneficial effect of PEMFs treatment is widely reported in different conditions, there is still a lack of knowledge about its mechanisms of action.

For this reason, the aim of this work was to investigate the molecular mechanisms involved in the positive effect of PEMFs therapy on both the survival of the neuronal cells and the regulation of the inflammation mediated by microglial cells.

In particular, this study led to the publication of two scientific articles on peer-review journals:

- "*Pulsed electromagnetic field and relief of hypoxia-induced neuronal cell death: The signaling pathway*" (https://doi.org/10.1002/jcp.28149);

- "*Signaling pathways involved in anti-inflammatory effects of Pulsed Electromagnetic Field in microglial cells*" (https://doi.org/10.1016/j.cyto.2019.154777);

that will be presented in the two following chapters.

CHAPTER 1

"Pulsed electromagnetic field and relief of hypoxia-induced neuronal cell death: The signaling pathway"

INTRODUCTION

PC12 CELL LINE: A NEURONAL MODEL FOR STUDYING HYPOXIA

PC12 cells are immortalized cells derived from a rat pheochromocytoma of the adrenal medulla (Green and Tischler, 1976). These cells appear small in dimensions (6-14 μm in diameter) and mostly round. Since their discovery, that reported they have a chromosome number of 40, these cells have been the object of intense investigations. They grow in standard medium in plastic surface petri or flask, to which they weakly attack, with a doubling time of 48/96 hours. By histofluorescence it was reported they contain catecholamine, dense core and chromaffin-like vesicles. The principal catecholamine present is dopamine (Green and Tischler, 1976; Perlman et al., 1982), followed by norepinephrine (differently from the adrenal medulla cells, where this is the major one) and epinephrine, that is present in traces or absent. It has been identified the presence of the enzymes for the synthesis of catecholamines (Green and Tischler, 1976). These cells contain acetylcholine (Greene and Rein, 1977b), to whom they are also sensitive, due to the presence of both nicotinic and muscarinic acetylcholine receptors, resembling those on sympathetic neurons (Patrick and Stallcup, 1977a, 1977b). The binding of the nicotinic one leads to the release of catecholamines (Greene and Rein, 1977a). A very expressed receptor type is the adenosine receptor (Guroff et al, 1981), in particular the A_2 (Noronha-Blob et al, 1986; Williams et al, 1987), and it has been reported also the presence of enkephalin receptors and of two different forms of Nerve growth factor (NGF) receptors, which differ on the rate of releasing the ligand from its site of binding. Although they possess voltage-dependent K + channels, the low density of the Na+ channels present characterises PC12 cells for their electrical in-excitability (O'Lague et al., 1980).

Therefore, when these cells are grown in a normal medium, their characteristic are specific for the adrenal cells. However, an interesting aspect is that they can be differentiated with NGF into sympathetic ganglion neurons, assuming their characteristics both functionally and morphologically (De Rios et al., 2017; Matsuzaki et al., 2019) (Fig.2).

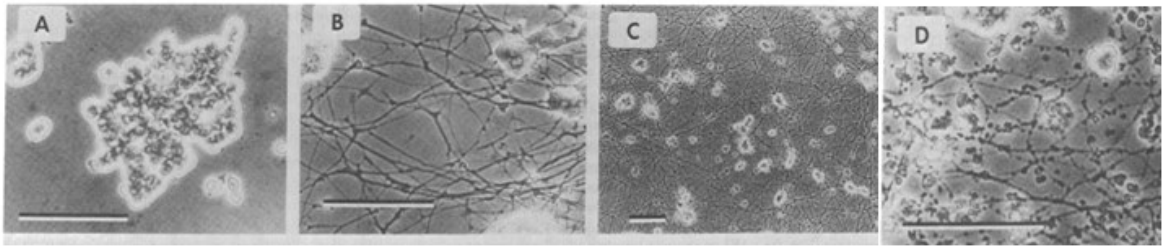

Figure 2. (A-D) contrast microscopy of PC12 cells: A) without NGF: B) after 14 days of NGF treatment; C) after 22 days of NGF treatment; D) 1 day after the removal of NGF, following 14 days of NGF treatment (Green et al., 1976).

NGF was the first member of the neurotrophin family to be discovered almost 70 years ago. In 1953, Rita Levi-Montalcini found out that fragments of a mouse sarcoma tissue, implanted in chick embryos, was able to stimulate the growth of nearby sensory and sympathetic ganglia (Levi-Montalcini, 1987). The responsible was then isolated, purified and named NGF, a peptide with molecular weight 26,000 (Cohen, 1960) that is now known to be a powerful and selective growth factor (Aloe et al., 2012).

In fact, experiments performed with the injection into neonatal rodents, of the antiserum derived from the use of NGF as an antigen, showed the permanent loss of the sympathetic nervous system (Levi-Montalcini and Booker, 1960), confirming its role in the maintenance of sympathetic neurons (Johnson et al., 1980).

From the original work, the target of NGF was reported to be the sympathetic and sensory neurons; however, sometime later, it was reported that this peptide could influence also the chromaffin cells of the adrenal medulla. Even if NGF is not necessary for these cells, it is able to promote, in adrenal medullary cells from young animals, both in vitro (Unsicker et al., 1978) and *in vivo* (Aloe and Levi-Montalcini, 1979), the elaboration of neurites and their conversion into sympathetic neurons.

In fact, PC12 cells treated with NGF starts to modify their morphological, biochemical and electrophysiological characteristics, to finally resemble sympathetic cells.

Under the microscope, it is evident the formation of branched, varicose processes and that they stop dividing (Fujita et al., 1989).

In fact, NGF activates the tropomyosin receptor kinase A (TrkA) that shift cells proliferation toward their differentiation, through the ribosomal S6 kinase 1 (RSK1). It was reported also an increase in the expression of the microtubule-associated protein 2 (MAP-2), that is involved in neuritogenesis (Lee et al., 2013), and an increased

phosphorylation of different cytoskeletal proteins, such as vinculin (Halegoua, 1987), some neurofilament proteins (Lindenbaum et al., 1987) and a 250,000 molecular weight, cytoskeletally associated protein (Landreth et al., 1985).

On the opposite, it was described a decrease in the levels of both the lactic acid dehydrogenase (Calissano et al, 1985; Cattaneo et al, 1985) and the epidermal growth factor receptor, that seems to be involved in the decreased cell proliferation NGF-induced (Huff et al, 1981; Lazarovici, et al., 1987; Boonstra et al., 1987).

It was demonstrated an increase of dopamine and enkephalin receptors (Inoue and Hatanaka, 1982) and an augmented sensitivity to acetylcholine. Upon treatment, cells showed electrical excitability, due to the presence of potassium channels (Wiatrak et al., 2020) and to the 10- to 20-fold increase in the density of Na + channels (Rudy et al., 1987).

Lastly, synapsin I, a protein involved in synaptic communication, in the release of neurotransmitters in synapses, in synaptogenesis and neuronal plasticity (Mirza et al., 2017), has been found in PC12 differentiated cells in a level dependent on cells differentiation (Das et al., 2004).

All these aspects indicate that differentiated PC12 cells possess neuronlike characteristics, however, the final validation was the demonstration of their ability to interact with myotubes from the clonal rat skeletal muscle line L6, with the formation of functional cholinergic synapses (Schubert et al., 1977).

The interesting thing is that differentiation is reversible; in fact, the remotion of NGF lead to the loss of processes and to cells proliferation (Fujita et al., 1989).

Over the years, different studies investigated the characteristic of this cell line, their proliferation and differentiation, creating a large amount of background knowledge. Because of this, and along with their easy manipulation to passage and culture, PC12 differentiated cells have become a popular and widely used model for the in vitro study of neurons (Wiatrak et al., 2020).

They have been utilised in studies of cell differentiation and survival (Miller and Kaplan, 2001; Vaudry et al., 2002; Agell et al., 2002; Anneren et al., 2003), apoptosis (Valavanis et al., 2001; Macdonald et al., 2003), but also neuronal disease such as Parkinson's (Elkon

et al., 2001; Ryu et al., 2002), Alzheimer's (Ge and Lahiri, 2002; Leutz et al., 2002) and Huntington's (Sipione and Cattaneo, 2001; Peters et al., 2002).

More, PC12 cells have been studied in depth in hypoxic condition and have been considered a valid model to study this condition and to obtain information about the molecular mechanisms involved (Hillion et al., 2005).

MEDIATORS INVOLVED IN CELL-SURVIVAL

- **Pathways regulated by the MAPKs**

Mitogen-activated protein kinases (MAPKs) are a group of serine-threonine kinases involved in many cellular activities such as: cell proliferation, differentiation, transformation, survival and death (McCubey et al., 2006; Torii et al., 2006; Dhillon et al., 2007). In mammals, this family is composed by extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK). Once activated, these kinases mediate different intracellular signaling, by phosphorylating numerus substrate proteins. In turn, they are activated by other kinases or by a complex of kinases guided by a scaffold protein.

In particular, JNK and p38 are triggered by pro-inflammatory cytokines (es the TNF-α or interleukin IL-1β) or in response to stressful events, such as hypoxic and oxidative stress (Fig. 3).

These signals are transduced into the cells by activating different MAP2Ks such as MKK3 and MKK6, that led to the activation of either p38 and themselves, that are triggered by the same MAP3Ks (such as ASK1 and TAK1) involved in the JNK pathway.

The receptor tyrosine kinase (RTK)-Grb2-SOS activates the small GTPase Ras, whose members transmit the extracellular signals into the cell (Malumbres et al., 2003), and subsequently Raf, which activates MEK1/2 and finally ERK and its pathway (Dhillon et al., 2007).

ERK pathway, that is triggered by cytokines, osmotic stress, growth factors and mitogens, is involved in cell proliferation and differentiation, neuronal plasticity, and cell survival; while JNK and p38 MAPKs are implicated in the modulation of inflammation, cell cycle arrest and apoptosis (Yamazaki et al., 2018).

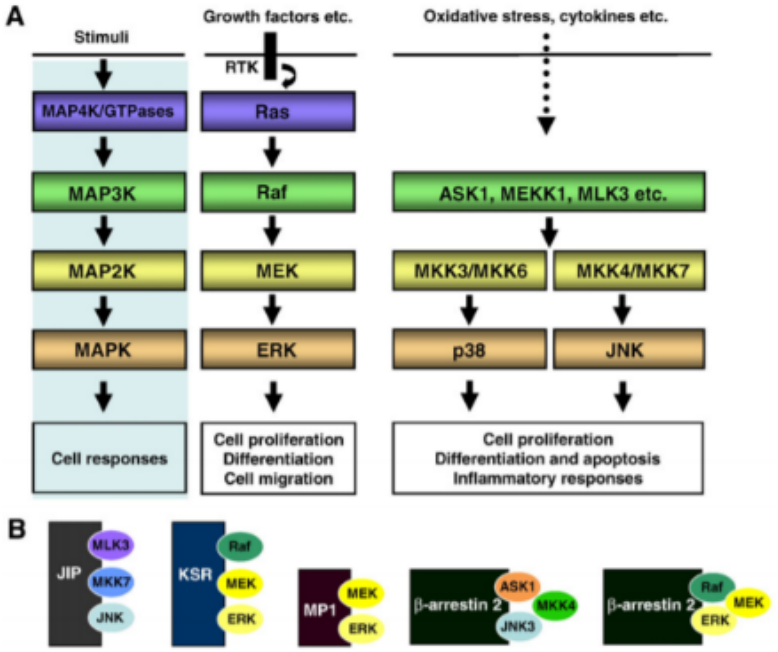

Figure 3. Mitogen-activated protein kinase (MAPK) signaling pathways. A. MAPK signaling pathways mediate intracellular signaling initiated by extracellular or intracellular stimuli. MAP3Ks, which are activated by MAP4Ks or GTPases, mediate phosphorylation and activation of MAP2Ks, which in turn phosphorylate and activate MAPKs. Activated MAPKs phosphorylate various substrate proteins including transcription factors, resulting in regulation of a variety of cellular activities including cell proliferation, differentiation, migration, inflammatory responses, and death. The mammalian MAPK family includes ERK, p38, and JNK. In the ERK signaling pathway, ERK1/2 is activated by MEK1/2, which is activated by Raf. Raf is activated by the Ras GTPase, whose activation is induced by RTKs such as the epidermal growth factor receptor. The p38 and JNK pathways consist of a MAP3K such as ASK1, MEKK1, or MLK3 as well as a MAP2K such as MKK3 or MKK6 for the p38 pathway or MKK4 or MKK7 for the JNK pathway. Activation by MAPK signaling cascades is achieved either through a series of binary interactions among the kinase components or through formation of a multiple kinase complex mediated by a scaffold protein. B. Scaffold proteins that facilitate activation of MAPK signaling pathways include JIP for the JNK signaling pathway, KSR and MP1 for the ERK signaling pathway, and β-arrestin 2 for the ERK and JNK pathways (Kim and Choi, 2009).

In recent studies in rats was demonstrated that limb ischemic preconditioning (LIP), a procedure that provoke a light stroke event in organs different from the heart and the brain, was able to protect the pyramid neurons located in the hippocampus from the death

induced by an ischemic event. More, it was shown that the activation of p38 MAPK was responsible of the tolerance LIP induced (Sun et al., 2006). However, the signaling pathway involved in this beneficial effect is still unclear.

It is known, though, that p38 signaling pathway activates the family of heat shock proteins (HSP), that includes HSP 30, HSP 60, HSP 70 and HSP 90. These molecules play an important role after stressful events, such as elevated temperature, heavy metals, trauma, ischemia and hypoxia (Vass et al., 1988), protecting the cells and stimulating their recovery and survival. In particular, among these, HSP70 has been reported to aid cells after a brain ischemic event (Aoki et al., 1993a, 1993b; Yagita et al., 2001), and seems to be involved in the induction of ischemic tolerance (Nakata et al., 1993; Yagita et al., 2001). Based on that, Sun et al. suggested that the brain ischemic tolerance induced by LIP is mediated by the activation of HSP70 stimulated by p38 MAPK (Sun et al., 2010).

- **Protective role of the HSP70 chaperone molecule in hypoxia**

HSP are a group of chaperones, ranging in size from 8 kDa to 150 kDa (Malago et al., 2005), highly conserved and mostly ubiquitous, whose principal function consists in supporting the folding of both newly synthesized and stress-induced denatured proteins. They also exert numerous cytoprotective functions (Brown, 2007; Kim et al, 2018) and are known to prevent the apoptotic cascade (Giffard and Yenari, 2004).

The production of HSPs is stimulated in the NS in different pathologies, such as in neurodegenerative diseases, epilepsy, trauma and in cerebral ischemia (Sharp et al., 2000) and their presence was spotted in different cell lines, such as neurons and glia (Kim et al., 2016). More, they are detected also in the extracellular environment, where they are secreted because of the neuronal cell death and where they act increasing the stress resistance of the surrounding stress-sensitive recipient cells (es neurons), and as signals of danger for the inflammatory cells (Weiss et al., 2002).

In particular, in the brain, it has been reported that, among all, the heat and the ischemic insult leads to an increased production of HSP70 (Brown, 2007). Because of this, HSP70 was largely studied.

In humans, the Hsp70 multigene family comprises Hsp70, Hsc70 and Grp78, that are nuclear, cytosolic and endoplasmic reticulum localized respectively, and the mithochondrial MtHsp75.

It is known that Hsp70 levels are regulated by growth (Wu et al., 1985) and stimulated in response to stress, such as in hyperthermia, oxidative and mechanical stress. It intervenes in the folding of the new polypeptides and proteins and in the prevention of their aggregation, in the refolding of aggregated or misfolded proteins, in the promotion of ubiquitination and degradation of misfolded ones and is also involved in protein translocation and interactions with signal transduction proteins (Turturici et al., 2011).

In addition to its chaperone functions, HSP70 has been reported for its anti-inflammatory properties, making this molecule an interesting target to be modulated for therapeutic benefit. Indeed, the increase of HSP70 induced before or after renal ischemia/reperfusion injury (IRI) was reported to protect kidney function (O'Neill and Hughes, 2013), to inhibit the pro-inflammatory IL-8 in respiratory epithelial cells and to protect intestinal cells against ischaemia and the related reperfusion injury (Malago et al., 2005).

Works on cerebral ischemia reported an increased level of this chaperone in the regions more resistant to the ischemic insult, raising question about its involvement in the damage or in the survival of the cells of the brain (Sharp et al., 2000). So, studies that specifically increased HSP70 levels were made, leading to the conclusion that it possesses a protective role in brain cells against the experimental insults, including ischemia (Wiss et al., 2002; Chen et al., 2003; Jo et al., 2006; Kim et al., 2013; Kim et al., 2016). On the contrary, its reduction resulted in the opposite effects (Kim et al., 2020).

HSP70 increase showed to modulate the inflammatory response also in a model of transient middle cerebral artery occlusion (tMCAO); in fact, transgenic mice overexpressing the chaperone resulted protected against brain ischemia (Zheng et al., 2008).

Similar results were showed in rat stroke models, where the increase of HSP70 levels, obtained by either transgenic overexpression (Planas et al., 1997; Plumier et al., 1997) or induced overexpression with either a Herpes or Adenoviral vector, led to a reduced global and focal ischemia brain injury (Giffard and Yenari, 2004). The neuroprotection was obtained also with the administration of a Herpes viral vectors, that induced Hsp70 expression in rat brain, 12 to 15 hours before a focal or global ischemia (Yenari et al.,

1998; Kelly et al., 2002) and 30 minutes and 2 hours after a 1-hour middle cerebral artery occlusion (Hoen et al., 2001). These results are important because the level of the proteins starts to be expressed by the vector from 4 to 6 hours after the injection, and reaches its peak after 24-48 hours, meaning that the protection afforded by HSP70 increase may represent a valid solution in the clinical setting, when patients are received after the ischemic event.

More, the HSP70 increase in murine astrocytes was reported to be protective from glucose and oxygen deprivation and H_2O_2 exposure, and in co-cultured neurons and astrocytes to be neuroprotective (Giffard and Yenari, 2004).

The decreased inflammatory response due to the increase of HSP70 may involve also microglial cells. It is known, in fact, that these cells are responsible of the release of cytotoxic and inflammatory mediators after brain ischemia (Block and Hong, 2005). Zheng and colleagues demonstrated that HSP70 increase in brain ischemia is able to modulate both their cytotoxicity and activation, downregulating several pro-inflammatory genes. More, the same study reported the implication of HSP70 in blocking NF-kB activation and so in modulating the expression of some downstream inflammatory genes (Zheng et al., 2008).

Overexpression of HSP70 has been reported to intervene in the process that activate matrix metalloproteinases (MMPs), preventing their transformation in the active form. In fact, cultured astrocytes overexpressing HSP70 subjected to ischemic insult was reported to have a reduced MMP-9 mRNA and MMP-2 expression, confirming that HSP70 can modulate inflammation also at the transcriptional level (Lee et al., 2004).

The anti-inflammatory results have been reported to be associated also to a decrease of cytokines and pro-inflammatory mediators. HSP70 augmented level, achieved through a genetic manipulation or heat stress treatment, led to a reduced production of NO and of its inducible synthase (iNOS) (Yenari et al, 2005), of reactive oxygen species (ROS), and to a decreased secretion of various pro inflammatory cytokines such as TNF- α, IL-1, IL-10 and IL-12 (Van Molle et al, 2002; Ding et al, 2001). Experiments on a model of intracerebral hemorrhage proven that HSP70 increase is able to preserve blood brain barrier (BBB), to reduce brain edema, and to improve neurological functions (Manaenko et al, 2010).

HSP70 has also been shown to mediate cytoprotection by interfering and regulating apoptotic and immune pathways (Kim et al., 2020).

One interesting study in experimental stroke models reported that HSP70 increases at first within the neurons of the penumbra (Sharp et al, 2000) or in the brain area near to the infarcted area and subsequently within astrocytes, microglial cells and endothelial cells of cerebral vasculature. This neuroprotective effect was reported also comparing the improved neurological outcome of transgenic mice, with an increased HSP70 expression, to the wild type after a cerebral ischemia. At the same time, similar models investigated the deficiency of this molecule, reporting an aggravated outcome (Kim et al., 2016; Lee et al., 2001).

These results raised the question whether HSP70 may also regulate the apoptotic pathway. In fact, different studies, investigating both the in vitro and the *in vivo* cells, observed that the one overexpressing HSP70, have an increased Bcl-2 level too (Giffard and Yenari, 2004).

- **CREB: activation, functions and effects in hypoxic conditions**

Cyclic AMP (cAMP)-responsive element-binding protein (CREB) is a leucine zipper transcription factor, which is known to be widely distributed among all the tissues (Yamashima, 2012). The cAMP response element (CRE) was first described in 1986 by Marc Montminy and R.H. Goodman as a defined DNA sequence located within the promoter region of various genes, activated by cAMP, that permit to control the genes expression. In fact, the RNA polymerase activity could be modulated through the binding of this region with different transcription factors. One years later, in 1987, Montminy and Bilezikjian individuated and described the transcription factor that, binding CRE, induced the transcription of the somatostatin gene, indicating it as CREB (Montminy and Bilezikjian, 1987).

To date, it is known that the CREB family, belonging to the superfamily of the leucine zipper transcriptional regulators, is composed of CREB, CRE modulator (CREM) and

activating transcription factor-1 (ATF-1), and mediates cAMP-responsive transcription (Ortega-Martinez, 2015).

Among the family, CREB and ATF-1 are ubiquitous, whereas CREM is expressed mostly in the neuroendocrine system. There is a single gene encoding for each transcriptional factor, which contains the sequence of the highly conserved leucine zipper and a basic region, responsible for the binding to the CRE DNA sequence (Barco et al., 2003).

The gene that encodes for CREB is Creb1, that can generate three isoforms; among all, the α is the most common, but the β and Δ isoforms are also transcribed. Experiments conducted in the absence of them demonstrated their involvement in long term memory (Bourtchuladze et al., 1994) and in the plasticity of the adult neocortex (Glazewski et al., 1999).

To exert its transcriptional activator action, CREB binds the CRE element as both a homodimer or a heterodimer, even if the first one is the most potent form (Dworkin and Mantamadiotis, 2010).

However, this mediator is inactive until it is phosphorylated. In detail, for CREB, its phosphorylation at Ser133, its transcription activating site, is performed by different serine-threonine kinases such as cAMP-dependent protein kinase A (PKA), PKC, MAPK and Ca2+/calmodulin-dependent protein kinase IV (CaMKIV) (Wen et al., 2010), while its dephosphorylation by protein phosphatase (PP)-1 and PP2-A (Ortega-Martinez, 2015).

Once phosphorylated, CREB and its coactivator protein, the CREB-binding protein (CBP) or p300, interact with each other, in order to allow the transcription of the responsive genes. In particular, CBP is involved in the modulation of the chromatin, acetylating histone and in recruiting factors necessary for RNA polymerization (Wen et al., 2010).

The game among the protein kinases and phosphatases determines the activation of the mediators, controlling different processes, such as the cell cycle (Liu et al., 2014), cell death (Martin, 2010), DNA damage (Abreu et al., 2013) neurogenesis (Faigle and Song, 2013), adaptive responses, glucose homeostasis, spermatogenesis, circadian rhythms, and synaptic plasticity associated with memory (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001; Sakamoto and Frank. 2009).

Further, evidence over the past decade demonstrated its role in the immune responses.

In particular, the interest in CREB activity in the nervous system has been increasing since its isolation in undifferentiated neuron-like PC12 cells (Montminy and Bilezikjian, 1987) and from the mouse brain (Yamamoto et al., 1988). Ever since, CREB role has been identified in cell survival, proliferation and differentiation in the developing brain and in learning, memory, and neuronal plasticity in the adult brain (Yamashima, 2012).

An augmented phosphorylation of CREB has been identified in different pathological conditions (Zaitseva et al., 2005), and both an increase in phosphorylation and in DNA-binding activity has been reported in global and focal ischemia (Yoneda et al., 1994; Salminen et al., 1995; Walton et al., 1996; Hu et al., 1999a; Tanaka et al., 1999a, 1999b; Walton et al., 1999). In particular, the increase was found in the dentate granule cells (global ischemia) (Walton et al., 1996; Hu, et al., 1999a) and in the ischemic boundary zone (focal ischemia) (Tanaka et al., 1999a, 1999b), that is associated with neuronal survival after the ischemic insults. Because of this, the protective effect of CREB was further investigated. It is known that CREB, once activated, interacts with CBP, that is involved in the signaling pathways exploited by hypoxia-inducible factor-1α (HIF-1α), erythropoietin and vascular endothelial growth factor (VEGF) (Arany et al., 1996). The same mediators are stimulated in cerebral ischemia with a final neuroprotective effect (Jin et al., 2010).

Walton and colleagues reported an increase in CREB levels in cortical and dentate granule cells, while vulnerable CA1 hippocampal cells lost CREB immunoreactivity after an ischemic insult (Walton et al., 1996). They also demonstrated that CREB upregulation could inhibit neuronal apoptosis (Walton et al., 1999).

After them, Jin and colleagues demonstrated that pCREB and CBP levels increased in CA1 hippocampal neurons after global cerebral ischemia and that, in an in vitro model of cerebral ischemia, CBP increase was concentrated in cortical neurons with intact nuclear morphology (Jin et al., 2000).

On the contrary, experiments performed in CBP mutant mice showed that the lack of this mediator led to embryonic death from cerebral hemorrhage and to defects in both neural and vascular morphogenesis (Tanaka et al., 2000). In addition, its sequestration is

responsible of an aberrant transcriptional regulation, a typical characteristic of neurodegenerative diseases (McCampbell et al., 2000; Steffan et al., 2000), confirming CBP importance in neuronal survival.
It was then assessed whether neuronal survival and plasticity may depend on pCREB and BDNF levels (Mohamed et al., 2019). In fact, among the downstream targets of CREB, some neurotrophic factors, such as BDNF (Tao et al., 1998), and antiapoptotic genes, like Bcl-2 (Riccio et al., 1999) have been identified.

Ischemic protection CREB mediated has been suggested to act through the induction of CRE-containing genes such as Bcl-2 as well as BDNF and its receptor trkB. In particular, Bcl-2, that will be described later, is an anti-apoptotic molecule, that offers protection against apoptosis and ischemic neuronal death, and BDNF has been reported to be neuroprotective in different in vitro and *in vivo* models of ischemia (Zaitseva et al., 2005).

Finally, Mohamed and colleagues demonstrated that, in neuronal cells, the increase of pCREB was accompanied with an increase in BDNF levels (Mohamed et al., 2019) supporting the results obtained in different I/R models in hippocampal cells (Jover-Mengual et al., 2007; Fu et al., 2013; Dong et al., 2014). Further, they reported a subsequent increased in GLUT3 expression, the main glucose transporter in neurons; asserting that these elements were able to guarantee neuronal survival in cerebral ischemia reperfusion (Mohamed et al., 2019).

- **Pathways triggered by BDNF and its importance in cerebral ischemia**

Nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) are a family of endogenous peptide known as neurotrophins (Wurzelmann et al., 2017).

NGF was the first to be discovered, in 1951, and owes its name to its trophic (growth promoting) effects on sensory and sympathetic neurons (Levi-Montalcini and Hamburger, 1951). The second one was the brain-derived neurotrophic factor (BDNF)

that was identified because of its positive effects on the survival of a subpopulation of dorsal root ganglion neurons (Barde et al., 1982). Since its purification from pig brain, other members have been described.

These molecules are secreted from neurons and glial cells and are involved in the regulation of various functions, such as survival and development of individual cells and neuronal networks across the entire brain, but also in the regulation of synaptic plasticity, in the neuronal protection from oxidative stress and apoptosis, and in neurogenesis (Skaper et al., 1998; Leal et al., 2015; Kuipers et al., 2016).

Among all, BDNF is one of the most studied because of its ubiquitous expression in the brain and its potent properties. To exert its function, this mediator interacts with two different specific receptors: p75NTR and TrkB; to whom it has a high affinity. In particular, the bind of p75NTR receptor is connected to apoptosis, while TrkB signaling is involved in neuronal survival, neuronal plasticity, and neurogenesis (Lu et al., 2008).

BDNF, in fact, has shown to have an important role in neurite and axonal growth (Yoshii and Constantine-Paton, 2010), and in the survival and development of different type of neurons (Pillai, 2008). More, through TrkB it modulates cognitive functions and is involved in both the processes of memory formation and consolidation (Zeng et al., 2012) by modulating adult neurogenesis and the survival of new-born neurons (Scharfman et al., 2005; Chan et al., 2008; Gao and Chen, 2009).

Other than the signaling pathways activated by the TrkB receptor itself, BDNF is also able to cause its dimerization and autophosphorylation, inducing its internalization that initiates a different intracellular signaling cascades (Levine et al., 1996) which includes the phosphatidylinositol-3-kinase (PI3K), the PLCγ, and the MAPK pathways. The first one led to the activation of Akt, which is known to promote cell survival through the inhibition of Bad and, consequently, allowing the expression of anti-apoptotic proteins (Yoshii and Constantine-Paton, 2010). In addition, Akt phosphorylation, and so activation, has been correlated to the suppression of pro-caspase-9 and Forkhead (Kaplan and Miller, 2007), that are other pro-apoptotic mediators, shifting the balance towards the anti-apoptotic pathway and resulting in attenuated cell death (Nathoo et al., 2004). The PLCγ pathway activate the inositol triphosphate (IP3) receptor, stimulating the release of intracellular calcium and the increase of calmodulin kinase (CamK) activity and

triggering CREB. In addition, MAPK pathways is related to cell growth and differentiation. So, while the PLCγ pathway mediates a quite fast response, the other two ways are involved in long-term transcriptional effects (Yoshii and Constantine-Paton, 2010) (Fig. 4).

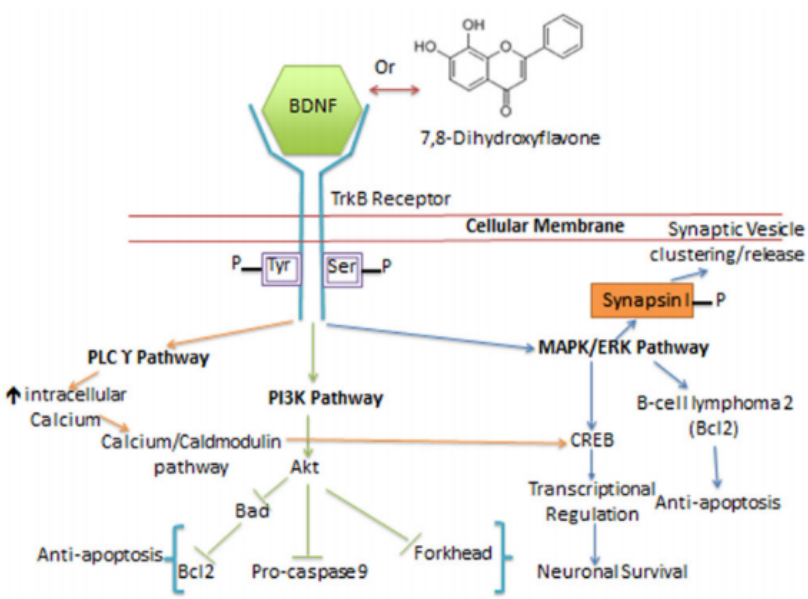

Figure 4. The activation pathways of the TrkB receptor. The binding of BDNF or 7,8-DHF leads to auto-phosphorylation of the intracellular domain of the receptor and activation of the downstream pathways. 1) The MAPK pathway. Activation of this pathway stimulates anti-apoptotic proteins, including Bcl2 and cAMP response-element binding protein (CREB). CREB is required by neurotrophin mediated neuronal survival. Activation of the MAPK pathway also stimulates extracellular signal related kinase (ERK), which induces phosphorylation of Synapsin I mediating the clustering and release of synaptic vesicles. 2) Activation of the PI3K pathway, which activates Akt. Akt inhibits apopotosis by inhibiting activation of antiapoptotic proteins including Bad, Pro-caspase 9 and Forkhead. PI3K = phosphatidylinositol 3 kinase; Akt = Protein Kinase B, PKB; Bad = Bcl2 associated death promoter. 3) The Phospholipase C-gamma (PLCγ) pathway. PLC-γ can lead to an increase in intracellular calcium levels, which activates the calcium/calmodulin pathway leading to CREB activation. 7,8-DHF: 7,8-Dihydroxyflavone; BDNF: brain-derived neurotrophic factor; MAPK: mitogen-activated protein kinase; TrkB: tropomyosin related kinase B (Wurzelmann et al., 2017).

The protection induced by BDNF increase have been reported also in cerebral stroke. In fact, the activation of the PI3K/Akt and MAPK/ERK pathways has shown to protect neurons from apoptosis (Liu et al., 2020). In order to obtain the BDNF increase, different strategies have been tested, such as the administration of sex hormones and antioxidants. A study in rat model of middle cerebral artery occlusion (MCAO) demonstrated that the

poststroke administration of progesterone reduces the apoptosis and cells injury through the PI3K/Akt pathway (Ishrat et al., 2012), and whether combined with vitamin D, it can protect neurons from the apoptosis induced by ischemia/reperfusion- (I/R-) injury by activating the ERK pathway and finally by both decreasing caspase-3 cleavage and increasing Bcl-2 production (Atif et al., 2013). Antioxidant administration has been reported to decrease I/R-induced apoptosis in neurons, too. In fact, BDNF increase stimulates the expression of Bcl-2 and the reduction of Bax, a pro-apoptotic mediator, resulting in less astrocytes' apoptosis (Wang et al., 2014).

In reperfusion injury, the oxidative stress leads to a deteriorate brain metabolism. Another proof of BDNF positive involvement in this context has been reported in SH-SY5Y line, a model for human neuronal cell. Their treatment with ethanol, in fact, significantly reduced the production of BDNF and CREB; on the contrary, in primary culture neurons from rat hippocampi, exogenous BDNF was able to reduce ethanol-induced damage (Chen et al., 2013).

For these reasons, different strategies to increased BDNF levels have been tried. Adeno-associated viral vector that promotes BDNF expression, injected in neurite cells in a serum deprivation condition, was reported to promote the cells outgrowth and to protect neurons from apoptosis (Zhang et al., 2011a). *In vitro* intraventricular infusion of BDNF significantly reduced the mean infarct if compared with control rats (Takeshima et al., 2011). Comparably, in rat induced global ischemia, BDNF administration increased the number of pyramidal cells. Injection of human BDNF after a H/R decreased the number of caspase-2-positive cells (Kurokawa et al., 1999), and in a postnatal day 7 rat model, BDNF was able to block the apoptotic stimulus caused by H/I injury, induced by caspase-3 activation (Han et al., 2000).

In hypoxic/ischemic injury, BDNF increase seems to have a role also in the inflammatory process. In fact, it was reported to promote both microglial proliferation and phagocytic activity in vitro (Zhang et al., 2003) and its own secretion from these cells. In addition, intranasal administration of BDNF in H/I rats was shown to decrease pro-inflammatory TNF-α and its mRNA expression and to increase the anti-inflammatory IL-10 protein (Jiang et al., 2011), that have been proven to exert an important function in damaging and protecting, respectively, cells in ischemia (McCoy and Tansey, 2008; Frenkel et al., 2009).

Other studies reported an essential role of BDNF in neurogenesis, comprising cell proliferation, migration and differentiation (Kuczewski et al., 2009). In fact, transplantation of BDNF-modified neural stem cells (NSCs) in a rat model of cerebral ischemia damage induced by temporary middle cerebral artery occlusion (tMCAO) had positive effects: BDNF protein was upregulated and there was neurite outgrowth in ganglion neurons (Zhu et al., 2011). BDNF was also suggested to sustain regenerative signaling at synaptic sites (Lessmann et al., 2003), to help in translating activity patterns into specific morphological changes (Horch et al., 1999) and to increase the expression of markers for axonal sprouting and synaptogenesis (Schabitz et al., 2004).

Because of its properties, BDNF was investigated as an agent for the treatment of stroke. It was reported that in rat MCAO, both infarct size and neuronal cells death was significantly reduced with an intraventricular or intravenous pretreatment of BDNF. More, it was also reported its positive involvement in neurogenesis, sensorimotor recovery, and neural plasticity (Schäbitz et al.,1997, 2004, 2004, 2007; Ferrer et al., 2001).

Therapeutic effects of BDNF on stroke were also evaluated investigating neurogenesis and migration. Positive results were obtained in a photothrombotic ischemia model by Schäbitz et al., who reported an increase in the numerosity of cells and in the migration of neural progenitor cells (NPCs) towards the striatum (Schäbitz et al., 2007) after an intravenous injection of BDNF (Schäbitz et al., 2007). In a mouse MCAO model, the increased BDNF level was positive too, augmenting the generation and the migration of neuronal precursor toward the ischemic area.

An increased BDNF level was reported to be significant in astrocytes too: trapping the mediator they contribute to both the neuronal precursor cells migration (Grade et al., 2013) and the increase of microglial cells, and so of cytokines and transcriptional factors, with a final protective effect and a decreased neuronal injury (Liu et al., 2020).

- **The anti- and pro-apoptotic mediators Bcl-2/BAD**

The apoptotic process, also known as programmed cell death, is an essential event that occurs in different phases of all multi-cellular organisms, such as in their development, adaptation and maintenance. One of its functions is to prevent the propagation of DNA damages caused by different mediators (Howells et al., 2011).

Cell death is regulated by mitochondria, an organelle responsible not only for the biochemical processes of respiration and energy production but also for cellular calcium homeostasis, ROS production and release of apoptogenic proteins. In detail, the major mitochondrial components include the outer and inner membranes, the respiratory complexes (I–IV) and the ATP synthase (complex V). Briefly, protons and molecules of hydrogen are pumped by complex I or II, complex III and complex IV from the matrix to the cytosolic side of the inner mitochondrial membrane. This process generates both the electrochemical gradient, that is involved in ATP synthesis and in calcium homeostasis, and the mitochondrial membrane potential (Ψm) (Murphy et al., 1999).

Mitochondria are damaged by ischemia (Siesjo et al., 1999): the lack of oxygen modifies the mitochondrial function and leads to the complete release of the cytochrome c, with the subsequent formation of a transition pore and finally the irreversible damage of mitochondrial components, that ends with cell death. It is known the involvement of the Bcl-2 family of proteins in regulating mitochondrial changes during both apoptosis and necrosis (Adams and Cory, 1998; Tsujimoto and Shimizu, 2000a).

The discovery of Bcl-2 was first reported thirty years ago, when an anomalous translocation between chromosomes 14 and 18 t(14;18) and a subsequent overexpression of Bcl-2 in acute B cell leukemia and follicular lymphoma cells was observed (Tsujimoto et al., 1984, 1985; Cleary and Sklar, 1985; Bakhshi et al., 1985; Cleary et al., 1986), contributing to cancer (Tsujimoto et al., 1985; Nunez et al., 1989). Because of that, it was discovered that its function was to increase cells survival, not through an increase in cell division rate but with the inhibition of apoptosis (Vaux et al., 1988; Tsujimoto, 1989). In particular, the term apoptosis (Gk: falling off, like a tree leaf) indicates a deliberate cell

death and was coined some years before Bcl-2 discovery (Kerr et al., 1972; Hockenbery et al., 1991).

Since then, different genes similar to BCL-2 have been identified, leading to the discovery of various proteins with pro- and anti-apoptotic role (Hata et al., 2015).
In particular, all the proteins produced possess at least one BCL-2 homology (BH) motif, a region composed of 10–20 amino acid with a great similarity across the Bcl-2 family. These motifs are four, and they are numbered in order of discovery (Hardwick and Soane, 2013).

Besides Bcl-2, the anti-apoptotic mediators are Bcl-xl, Bcl-w, Mcl-1 and Bfl-1/A1, that share structural homology with Bcl-2 in the BH 1, 2, 3 and 4 domains. Differently, the pro-apoptotic mediators, that are Bad, Bim, Puma, Bid, Bik, Bmf, Hrk and Noxa, share homology only in the BH3 domain, that is necessary for their role. In fact, they can provoke apoptosis in the so-called indirect way, introducing their BH3-containing helix into a specific hydrophobic site of the Bcl-2 proteins. They can also lead to the same result by binding and activating Bak and Bax, which form pores in the mitochondrial membrane leading to the release of the cytochrome c into the cytosol (Danial and Korsmeyer, 2004).

In particular, among the pro-apoptotic mediator, Bad is known for exerting its function in different way. In an anti-apoptotic condition, Bad is phosphorylated by 14-3-3, a molecule that bind phosphoserine. With its serine phosphorylated, Bad is isolated in the cytosol (Elmore, 2007). On the contrary, when unphosphorylated, it can lead to cell death by dimerizing with both Bcl-2 and Bcl-xl, displacing the two anti-apoptotic mediators from Bax, that becomes free, and binding Bax, leading to the formation of a dimer (Yang et al., 1995).

Different stimuli lead to the interaction between pro- and anti-apoptotic proteins, deciding cell destiny (Villunger et al., 2011).

Programmed cell death is responsible for various necessary processes such as the development of the embrio and the immune and nervous system, blood cell maturation and tissue homeostasis (Strasser et al., 2011). However, the deregulation of this family of

proteins has been involved in tumor formation and in the death associated to other conditions (Ouyang and Giffard, 2014).
For example, during cerebral ischemia or in the reperfusion-injury, the release of apoptosis-related proteins leads to a pathological change that results in nerve cells apoptosis. This event occurs in all the brain region that undergo the lack of oxygen, causing an irreversible damage which is reflected in motor dysfunction and neurological impairment (Liu et al., 2013).

Different studies reported a decreased active Bcl-2 level in cells that undergo apoptosis after cerebral ischemia/reperfusion injury (Li et al., 2009a, 2009b). In cerebral tissues of patients, the decrease is associated to an increased Bax homodimerization, that lead to an augmented cell apoptosis and a consequent loss of nerve functions (Youle and Strasser, 2008; Wang et al., 2012). In serum of patients with acute cerebral infarction, the expression of Bcl-2 has been reported to be reduced too (Salakou et al., 2007; Liang et al., 2014), suggesting that the restoration of the normal condition could protect the cells in the penumbra.

On these bases, the increase of Bcl-2 was tested, and a positive result in blocking the apoptotic effect after cerebral ischemia has been reported (Linnik et al., 1995; Asoh et al., 2002; Zhao et al., 2003). Studies in experimental stroke demonstrated the Bcl-2 protective effect against neuron loss in the ischemic margin, through the inhibition of the cytochrome c translocation (Zhao et al., 2003), and CA1 pyramidal neurons survival was increased in transgenic mice overexpressing Bcl-2 after transient global ischemia (Erfani et al., 2019).

In rats with acute cerebral infarction, Zhang et al. reported a reduced cerebral apoptosis, cerebral infarction range and nerve function damage due to Bcl-2 overexpression (Zhang et al., 2015).

The beneficial effects of Bcl-2 seem to be mediated by its ability to block the pro-apoptotic members by heterodimerization and to regulate the F1F0- ATPase/H+ pump (Matsuyama and Reed, 2000), the voltage-dependent anion channel (Tsujimoto and Shimizu, 2000b) and the adenine nucleotide translocator (Vieira et al., 2000), present on the outer and the inner mitochondrial membrane, respectively. In particular, it is reported

that Bcl-2 is associated to the calcium increase in mitochondria, reducing the respiratory damage (Murphy and Fiskum, 1999).

The protective effect mediated by the Bcl-2 family of anti-apoptotic mediators may depend also on their action on the endoplasmic reticulum (ER) membranes. The association between the ER and mitochondria, known as the mitochondria-associated ER membrane (MAM), is responsible of the Ca2+ transmission toward the second one (Ouyang and Giffard, 2012). Among all the proteins that participate in the communications inside the MAM, a central role is performed by the inositol 1,4,5-trisphosphate receptor (IP3R). It has been demonstrated that both Bcl-2 and Bcl-xl form a protein complex with the IP3R (Chen et al., 2004a, White et al., 2005), suggesting their involvement in its opening and so in the regulation of the Ca2+ flow (Fig. 5).

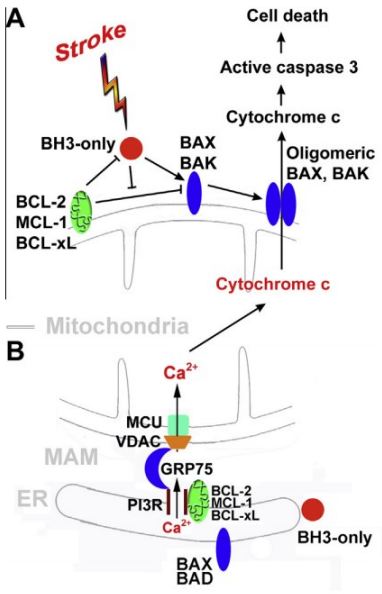

Figure 5. BCL-2 family-mediated cell death. (A) The BCL-2 family regulates mitochondria-dependent apoptosis. BH3-only family members act as sentinels for many death stimuli including ischemia. They can mediate the activation of the multidomain pro-apoptotic BAX and BAK allowing oligomerization. Upon BAX and BAK oligomerization, the mitochondrial outer membrane is permeabilized releasing a apoptogenic proteins from the mitochondrial intermembrane space, such as cytochrome c, into the cytosol. Released cytochrome c triggers the activation of a downstream caspase cascade leading to cell death. BH3-only proteins can be sequestered by anti-apoptotic BCL-2 family members (e.g. BCL-2, MCL-1, BCL-xL, etc.). (B) The BCL-2 family regulates ER-mitochondria Ca2+ crosstalk at the MAM. The basic structure for release of Ca2+ at MAM is the IP3R on the ER, and VDAC and MCU on the mitochondrion. IP3R and VDAC are physically coupled by the chaperone GRP75/mortalin. Excessive increases in mitochondrial matrix Ca2+ triggers opening of the mitochondrial outer membrane permeability pore causing the release of cytochrome c and other pro-apoptotic factors into the cytoplasm. ER: endoplasmic reticulum; GRP75: glucose-regulated protein 75/mortalin; IP3R: inositol trisphosphate receptor; MAM: mitochondria-associated ER membrane; MCU: mitochondrial Ca2+uniporter; VDAC: voltage-dependent anion channel (Ouyang and Giffard, 2014).

AIM OF THE STUDY

PEMFs treatment has been reported to have different positive effects on numerous biological systems. In particular, its beneficial role has been demonstrated in union and nonunion fractures, thus promoting the regeneration of the tissue in mammals (Qiu et al., 2020).

Interestingly, PEMFs has demonstrated to be also neuroprotective, exerting an anti-apoptotic effect against ischemic cell death, thus emerging as a potential alternative to the pharmacological protocols in ischemic diseases (Grant et al., 1994; Li et al., 2014; Pena-Philippides et al., 2014). However, the molecular pathways stimulated by PEMFs treatment to induce neuronal survival and to decrease cells apoptosis are still unknown.

As a consequence, it appears important to characterize the molecular mechanisms involved in the protective effects of PEMFs that have not been elucidated yet. 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 MAPK, Akt, and caspase-3 in neuron-like PC12 cells exposed to hypoxia have been examined in this study. Moreover, to better investigate the molecular actors involved in neuronal cells protection induced by PEMFs, the regulation of HSP70, CREB, BDNF, and the Bcl-2 family proteins in PC12 cells differentiated with NGF exposed to hypoxia was further explored.

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 system were from PerkinElmer (Milan, Italy). PKC-ε translocation inhibitor peptide was purchased by Calbiochem (Milan, Italy). Bad and Bcl-2 enzyme-linked immunosorbent assay (ELISA) kits were from LSBio (Seattle, WA). Quantikine ELISA BDNF immunoassay was from R&D System (Minneapolis, MN). 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 Dulbecco's modified Eagle medium (DMEM) F12 medium (Invitrogen, Grand Island, NY) supplemented with 5% fetal bovine serum (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% CO2) 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 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% CO2 (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 (De Mattei et al., 2009; Fini et al., 2013; Ongaro et al., 2012; Varani et al., 2002, 2003, 2008; 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 the peak intensity of the induced electric voltage were detected in the 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 the 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

PC12 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 signaling 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 ASSAY

Adenosine triphosphate (ATP) is a marker for cell viability: it is present in all metabolically active cells and its 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 AND TOTAL p38 MAPK ASSAY

AlphaScreen SureFire p-p38 (pThr180/Tyr182) and total p38 MAPK assays kit (PerkinElmer) were 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 a 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's instructions. Finally, fluorescent signals were detected through an Ensight PerkinElmer-multimode plate reader (PerkinElmer). 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). In brief, 150,000 PC12 cells were plated in six-well dishes and allowed to attach overnight. The subsequent morning, the medium was changed into a fresh one, in the

presence of a solvent or p38 inhibitor. After 24 hr 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 a mean ± standard error 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 (PerkinElmer), according to the manufacturer's instructions. PC12 cells were seeded in 100 µl culture medium into 96-well plates, allowed to attach overnight, then the medium was changed into a fresh one. After 24 hr of incubation in normoxic and hypoxic conditions and with or without PEMFs, cells were lysed and 10 µl of the lysate was transferred to a 384-well Optiplate™ for the assay. After the addition of both Acceptor and Donor mix solutions, fluorescent signals were detected through an Ensight PerkinElmer multimode plate reader (PerkinElmer). 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 PC12 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 hr in normoxic and hypoxic conditions and with or without PEMFs. A total of 50 µl of the supernatants was then put in the coated plate supplied by the kit and the levels of BDNF were searched out accordingly to manufacturer's instructions. Briefly, after 2 hr of incubation, each well was washed and then the total BDNF conjugated 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 PC12 cells were determined by Bcl-2 and Bad ELISA kits (LSBio). Briefly, 2,500,000 PC12 cells were seeded in small petri dishes and allowed to attach overnight. The day after the medium was changed and the cells were incubated for 24 and 48 hr in normoxic and hypoxic conditions and with or without PEMFs. Cells were then detached and lysed with three cycles of freeze and thaw. After 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 hr, respectively. Then, the 1X biotinylated Detection antibody solution was added. After 1 hr of incubation, each well was washed three times and then the 1X HRP conjugate 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 of independent experiments. Data sets were examined by one-way analysis of variance (ANOVA) and Dunnett's test (when required). A p-value of less than 0.05 was considered statistically significant.

RESULTS

Signaling 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 hr 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 signaling triggering this effect as well as the molecules modulated by PEMFs to reduce 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 hr in serum-free medium, in the absence and in the presence of PEMFs. To certify the involvement of these signaling 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 signaling 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 signaling pathways was triggered by PEMFs to exert their protective effects (Fig. 6). 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 hr, 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 signaling pathways did not affect cell death induced by hypoxia, while the 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 to exert their beneficial increase in cell survival (Fig. 7).

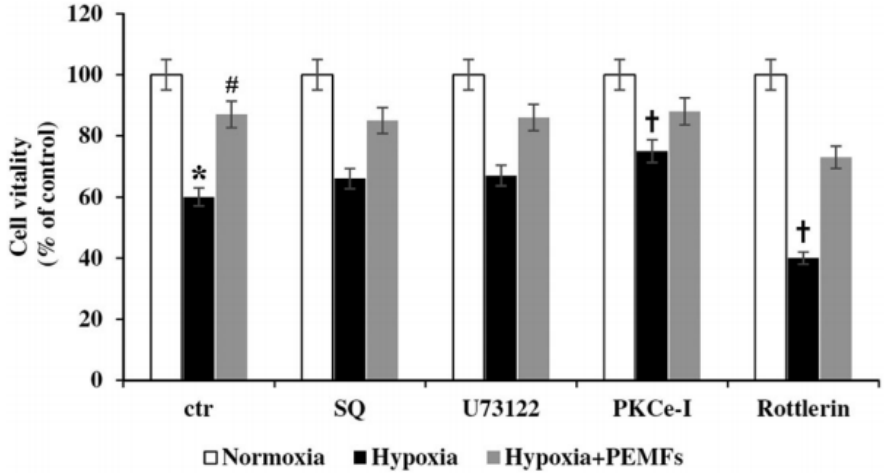

Figure 6. Effect of inhibitors of AC (SQ), PLC (U73122), PKC-ε (PKC-εI), PKC-δ (Rottlerin) at 1 μM on hypoxia-induced cell vitality in neuronal-like PC12 cells NGF-differentiated, in the absence/ presence of PEMFs. *$p < 0.01$ versus normoxia in the absence (ctr) or in the presence of each inhibitor; # $p < 0.01$ versus hypoxia in the absence (ctr) or in the presence of each inhibitor; † $p < 0.01$ versus control in hypoxia. AC: adenylyl cyclase; NGF: nerve growth factor; PC12: pheochromocytoma; PEMFs: pulsed electromagnetic fields; PKC-ε: protein kinase C epsilon; PLC: phospholipase C. Analysis was by one way ANOVA followed by Dunnett's test.

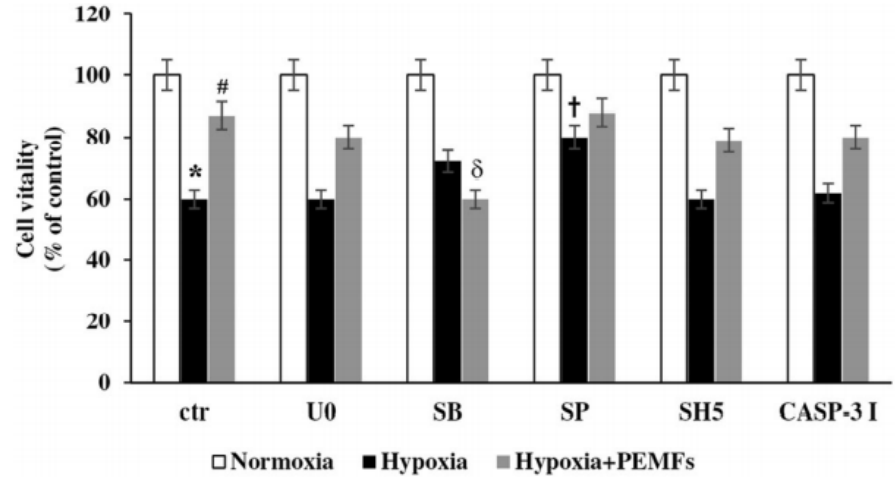

Figure 7. Effect of inhibitors of ERK1/2 (U0), Akt (SH5), JNK1/2 (SP), and p38 (SB) at 1 μM, caspase 3 (CASP-3 I) at 10 μM on hypoxia-induced cell vitality in neuronal-like PC12 cells NGF- differentiated, in the absence/presence of PEMFs. *$p < 0.01$ versus normoxia in the absence (ctr) or in the presence 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. NGF: nerve growth factor; PC12: pheochromocytoma; PEMFs: pulsed electromagnetic fields. Analysis was by one way ANOVA followed by Dunnett's test.

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, and 30 min) under hypoxia, in the absence and in the presence of PEMFs. As shown in Figure 8, 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 signaling pathway activated by them (Fig. 8).

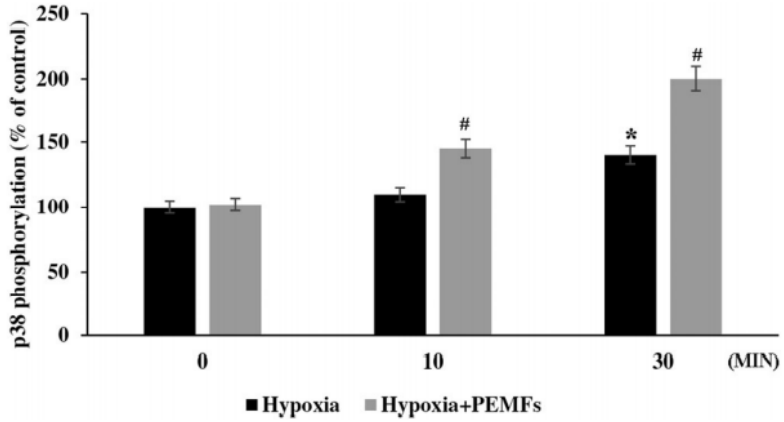

Figure 8. Kinetic effect of PEMFs on p38 MAP kinase activation at 0, 10, and 30 min in neuronal-like PC12 cells NGF-differentiated in hypoxia. *p < 0.01 versus hypoxia; # p < 0.01 versus hypoxia at each time point. MAP: mitogen-activated protein; NGF: nerve growth factor; PC12: pheochromocytoma; PEMFs: pulsed electromagnetic fields. Analysis was by one way ANOVA followed by Dunnett's test.

Effect of PEMFs on HSP70 levels in hypoxia

To investigate the neuroprotective mechanism of PEMFs, we explored whether the alterations of 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 hr and HSP70 levels were evaluated. HSP70 levels were not altered by hypoxia and hypoxia in the presence of PEMFs until 16 hr of treatment. However, we found a slight (114%) stimulatory effect by hypoxia alone after 24 hr of incubation in serum-free medium. In these conditions (24 hr hypoxia) cells exposed to PEMFs showed a significant stimulatory effect on HSP70 (140%) (Fig. 9). 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 stimulate HSP70 protein to induce neuroprotection (Fig. 9).

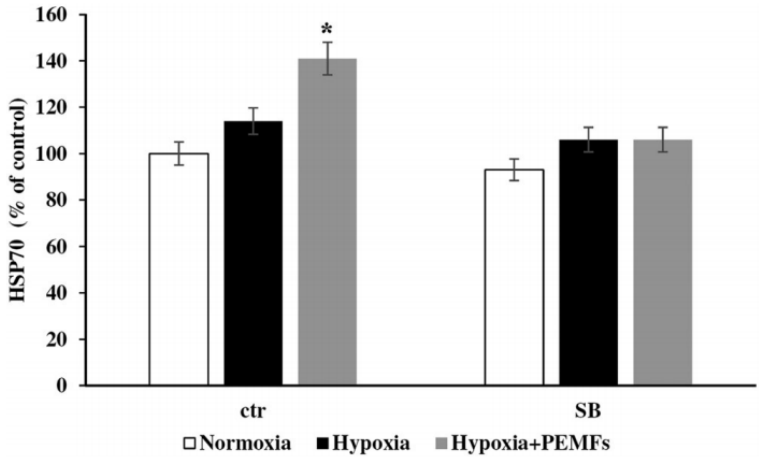

Figure 9. Effect of a p38 inhibitor (SB 1 μM) on HSP70 levels in neuronal-like PC12 cells NGF-differentiated in normoxia and hypoxia, in the absence/presence of PEMFs. *p < 0.01 versus control in hypoxia (ctr). *p < 0.01 versus hypoxia. HSP70: heat-shock proteins of 70; NGF: nerve growth factor; PC12: pheochromocytoma; PEMFs: pulsed electromagnetic fields. Analysis was by one way ANOVA followed by Dunnett's test.

CREB phosphorylation and BDNF regulation by PEMFs in hypoxia

In addition, we evaluated the ability of PEMFs to modulate CREB, a transcription factor downstream HSP70 as well as BDNF, a regulator of neuronal cell survival. Our results show that 24 hr of hypoxia alone induced a low increase of CREB phosphorylation, while cotreatment with PEMFs significantly increased it at the same time of incubation (Fig. 10). In addition, at 24 hr of hypoxia, we found a significant decrease of BDNF levels (40%), but PEMFs cotreatment did not affect them (data not shown). Instead at 48 hr of hypoxia, the decreased BDNF levels were restored to basal by PEMFs (Fig. 10). Pretreatment of PC12 cells with SB 1 μM for 30 min abrogated CREB and BDNF increase induced by PEMFs, suggesting that PEMFs trigger p38 stimulate CREB phosphorylation and BDNF increase (Fig. 10).

Pro-/Antiapoptotic 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 11 reports that hypoxia increased Bad after a treatment of 24 hr and PEMFs incubation significantly decreased this effect. At 48 hr its level was 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 level following a period of incubation of 24 and 48 hr, but PEMFs significantly increased it after 48 hr of cotreatment (Fig. 11). 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 modulate Bad and Bcl-2 in hypoxia (Fig. 11).

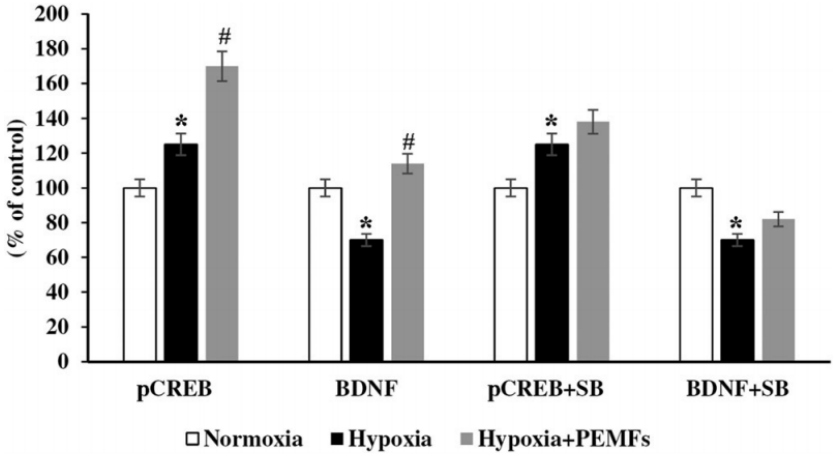

Figure 10. Effect of PEMFs on CREB phosphorylation (24 hr) and BDNF modulation (48 hr) in normoxia and hypoxia, in the absence and in the presence of the p38 inhibitor (SB 1 μM), in neuronal-like PC12 cells NGF-differentiated. *p < 0.01 versus normoxia; # p < 0.01 versus hypoxia. BDNF: brain-derived neurotrophic-factor; CREB: cAMP response element-binding protein; NGF: nerve growth factor; PC12: pheochromocytoma; PEMFs: pulsed electromagnetic fields. Analysis was by one way ANOVA followed by Dunnett's test.

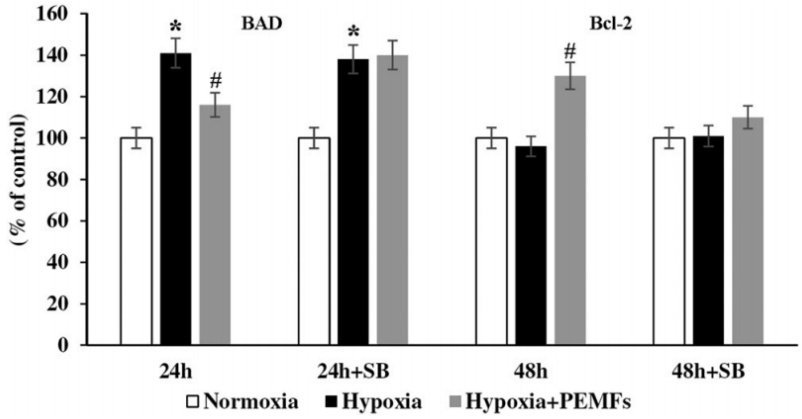

Figure 11. Effect of PEMFs on Bad and Bcl-2 (24 and 48 hr) modulation in normoxia and hypoxia, in the absence and in the presence of the p38 inhibitor (SB 1 μM), in neuronal-like PC12 cells NGF-differentiated. *p < 0.01 versus normoxia at each time; # p < 0.01 versus hypoxia at each time. NGF: nerve growth factor; PC12: pheochromocytoma; PEMFs: pulsed electromagnetic fields. Analysis was by one way ANOVA followed by Dunnett's test.

DISCUSSION

According to the World Health Organization, over 15 million people worldwide, every year, suffer a stroke (Ezzelarab et al., 2020). This disease is one of the world's biggest killer and the most frequent cause of long-term disability. It was calculated that 40% of the patients affected do not regain independence (Allen and Bayraktutan, 2009).

In particular, ischaemic stroke, that is the prevalent type, results from the interruption or the acute reduction of blood flow, and so oxygen, in cerebral arteries. In details, in the affected region two different areas can be distinguished: the core, where there is an instant neural death, and a surrounding area called penumbra, moderately hypoperfused, that keeps structural integrity but with a decrease or loss of function (Phan et al., 2002).

To date, the only available treatments for ischemic stroke patients are the antithrombotic therapies and the cerebral artery recanalization (Jauch et al., 2013). However, the outcome is not always positive: the vessel recanalization treatment could be successful, saving the tissue, or lead to no-reflow in the ischemic tissue bed, or result as deleterious causing cell death or hemorrhagic transformation. In fact, the subsequent restoration of blood flow in the damaged area, not only provides again oxygen, but is often associated to an increased injury of the affected tissue (Nour et al., 2012).

In this context, the therapeutical approach with PEMFs could represent a non-invasive solution. PEMFs has shown to exert several beneficial effects in cell biology, improving clinical outcome of regenerative medicine (Cadossi et al., 2014; Collarile et al., 2018).

In detail, PEMFs treatment has shown to stimulate bone repair in animal (Qui et al., 2020) and to modulate bone and cartilage metabolism, promoting chondrocyte and/or osteoblast proliferation and the production of the extracellular matrix components (Chalidis et al., 2011).

It was demonstrated that PEMFs can increase the production of growth factor beta-2 (Tepper et al., 2004), collagen I, anti-inflammatory prostaglandins and the VEGF-A-mRNA transcription by fibroblasts (de Girolamo et al., 2013), thus promoting angiogenesis, an important process involved in healing.

PEMFs treatment was discovered beneficial also in hypoxia related conditions. In human renal proximal tubular cells, PEMFs decreased the cell induced death by diminishing the

ROS production (Varani et al., 2017) and, in rabbits, the infarct size dimension was reduced after transient focal ischemia (Grant et al., 1994).

These results have been replicated also in mice, where PEMFs showed to decrease the infarct size and to exert anti-apoptotic effects (Pena-Philippides et al., 2014). PEMFs treatment was also tested in healthy humans by Capone and colleagues, that reported its tolerability and safety, not recording any side effects (Capone et al., 2009) and in a group of patients with acute ischemic stroke, in which there was a progressive improvement in clinical conditions and a decrease in the volume of infarct (Capone et al., 2017).

These results, together with other evidence from animal models of cerebral ischemia, led to the introduction of PEMFs treatment in a clinical multicentre, prospective, randomized, placebo-controlled, and double-blind study, whose aim is to investigate the effectiveness of PEMFs in acute ischemic stroke, as non-invasive and safe tool to gain neuronal recovery (Grant et al., 1994; Pena-Philippides et al., 2014; Capone et al., 2017; NCT02767778 clinicaltrial.gov).

However, the data regarding the pathway activated by PEMFs to exert their beneficial effects are still only few, underlying the need to carry out other studies.

In particular, this work characterised the pathway mediated by PEMFs to protect PC12 cells from apoptotic death under hypoxic condition. The neuroprotective effect of PEMFs, already demonstrated on neuronal viability, was confirmed in PC12 cells, and it was discovered an involvement, in this protective effect, of p38 MAPK. On the contrary, AC, PLC, PKC-ε, PKC-δ, ERK1/2, JNK1/2, Akt and caspase-3 pathways were not involved.

The phosphorylation of this kinase started soon after the PEMFs exposition, with a maximum level reached after 30 minutes.

Interestingly, p38 MAPK was not involved in the neuronal cell dead pathway stimulated by hypoxia, but it was triggered by PEMFs to reduce hypoxia-induced cell death. In the literature, there are contrasting evidence regarding p38 role; the result of this work is in line with the ones reporting a beneficial function of p38 in intracerebral hemorrhage, neurodegeneration, apoptosis and oxidative stress (Back et al., 2015; Xu et al., 2015; Yan et al., 2017; Gu et al., 2018).

Further, p38 MAPK involvement was reported in rats by Sun and colleagues, that described its role in the induction of tolerance by limb ischemic preconditioning to lethal brain ischemic insult (Sun et al., 2006) and later, in the protection of the pyramidal neurons in the CA1 region of the hippocampus, reporting a positive involvement of p38 MAPK against the neuronal death that is usually induced by ischemia (Sun et al., 2010).

For the first time, this study reported that PEMFs mediates the activation of HSP70, a chaperone molecule exerting strong cytoprotective effects in the ischemic brain, and that its activation is regulated by p38 MAPK. This protein increase is known for protecting the brain from ischemic damage (Rajdev et al., 2000; Tsuchiya et al., 2003; Kacimi and Yenari, 2015) and for its broad cytoprotective effects; in fact, it has been studied a lot as a possible therapeutic target to increase (Kim et al., 2014). To achieve this result, PEMFs seem to be a valid strategy.

It is known that MAPKs activate CREB, a transcription factor whose role has been identified in cell survival, proliferation, and differentiation in the developing brain and in learning, memory, and neuronal plasticity in the adult brain (Yamashima, 2012).

Because of this, the possible involvement of CREB/BDNF signaling pathway was evaluated.

Interestingly, PEMFs was found to increase CREB through p38 MAPK activation. This result is in line with the one obtained by Urnukhsaikhan and colleagues in human BM-MSCs (Urnukhsaikhan et al., 2016).

CREB increase is able to modulate BDNF, a mediator well known for its role in neuronal cell survival and differentiation, formation of the synapses and their plasticity and activity-dependent changes in synaptic structure and function (Park and Poo, 2013).
Thus, BDNF involvement in PEMFs mediated protective effects was investigated, and it was reported that p38 MAPK activation induced by PEMFs is able to restore the hypoxia-reduced BDNF level. The same neuroprotective pathway PEMFs mediated has been reported in mice PEMFs-treated after ischemic stroke (Li et al., 2014; Urnukhsaikhan et al., 2017).

In addition to BDNF, other downstream targets of CREB are some antiapoptotic genes, like Bcl-2 (Riccio et al., 1999). Ischemic protection CREB mediated has been suggested to act through the induction of CRE-containing genes such as Bcl-2 as well as BDNF and

its receptor trkB. In particular, Bcl-2 is an anti-apoptotic molecule, that offers protection against apoptosis and ischemic neuronal death (Zaitseva et al., 2005).

In fact, one of the major factors contributing to neuronal cell death is the cascade of molecular events associated with apoptosis.

It is known that mitochondria are damaged by ischemia (Siesjo et al., 1999): the lack of oxygen modifies their functions and leads to the complete release of the cytochrome c, with the subsequent formation of a transition pore and finally the irreversible damage of mitochondrial components, that ends with cell death. Bcl-2 family of proteins are known to regulate mitochondrial changes during both apoptosis and necrosis (Adams and Cory, 1998; Tsujimoto and Shimizu, 2000a). Therefore, it was examined the role of PEMFs to modulate Bcl-2, as the major anti-apoptotic, and Bad as a pro-apoptotic mediator in PC12 cells under hypoxic condition.

In particular, it was observed that hypoxia increased Bad, while it did not alter Bcl-2 level. On the contrary, PEMFs cotreatment was able to significantly decrease Bad expression stimulated by hypoxic condition, and to increase Bcl-2. These results suggest that PEMFs protection on neuronal death may also be due to the modulation of the mitochondrial apoptosis. In fact, the same pathway has already been observed in cardiac cells, in which PEMFs were able to reduce the death after myocardial ischemia/reperfusion injury (Ma et al., 2016).

So, to resume, the main findings of this study provide, for the first time, the indications that PEMFs are able to protect neurons from hypoxic-associated death, through the rapid activation of p38 MAPK, that leads to the increase of HSP70, CREB, BDNF and the modulation of the apoptotic proteins, increasing the anti-apoptotic Bcl-2 and decreasing the pro-apoptotic Bad (Fig. 12).

This study may invite further examinations on the molecular mechanisms of PEMFs in promoting neuronal cell survival, thus contributing to better understanding their therapeutical usefulness in CNS injured by hypoxic conditions, as happens during cerebral ischemia.

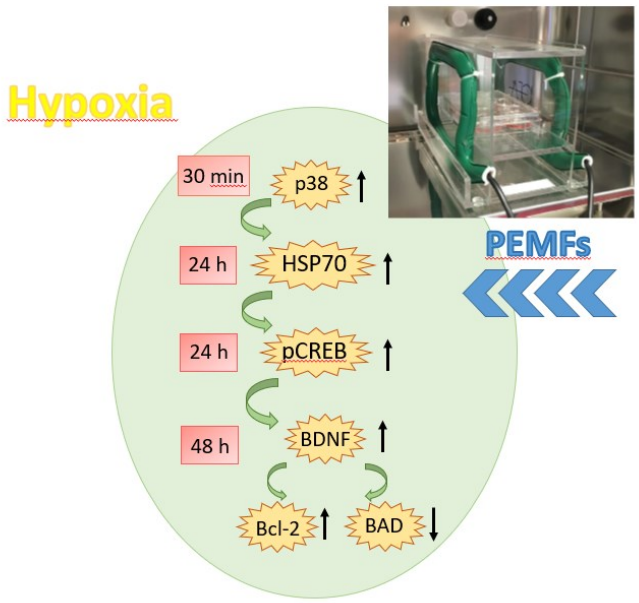

Figure 12. Graphical representation of the molecular pathway activated by PEMFs to decrease the neuronal cells' apoptosis. The pp38 MAPK increase leads to the activation of HSP70, pCREB, BDNF and of the apoptotic family of proteins, increasing Bcl-2 and decreasing BAD.

CHAPTER 2

"Signaling pathways involved in anti-inflammatory effects of Pulsed Electromagnetic Field in microglial cells"

INTRODUCTION

MICROGLIAL CELLS: origin, functions and role in ischemia

o ORIGIN OF MICROGLIAL CELLS

Microglial cells are the mononuclear phagocytes resident in the CNS and belong to the glial system of non-neuronal cells with supportive and protective functions. These cells are broadly disseminated in the brain and in the spinal cord (Lawson et al., 1990); they represent the 5-12% of the total glial cell population in the adult murine CNS, and the 0.5% to 16.6% of the total of cells in the human brain, depending on the region analysed. A similar regional variability has been found among rodents and humans, however, in human brain microglia shows higher density in the white matter than in the grey one (Mittelbronn et al., 2001).

In the healthy adult brain, microglial cells distribution is affected by the local cytoarchitecture (Tremblay et al., 2011). For parenchymal microglia, the general arrangement in a regular fashion that shows little cell layer or blood vessel pattern dependency was reported. However, it appears a tendency to avoid the cell dense fields such as granule cell regions. It is known that microglia density varies considerably across brain regions: in fact, while up to 12% of substantia nigra cells are microglia, they are only the 5% in corpus callosum. According to the regions, microglia morphology varies considerably too: white matter microglia present elongated soma and processes preferentially oriented along fiber tracts, in the region characterized by a leaky blood–brain barrier microglia show a compact morphology with few short processes and gray matter microglial cells present different radially oriented arbors (Lawson et al., 1990). The molecular reasons behind the anatomical diversity are largely unknown.

Microglia are considered the innate immune cells of the brain performing the primary immune surveillance and macrophage-like activities, such as the production of cytokines and chemokines (DiSabato et al., 2016), and the defence against infections or toxic substances produced by dying brain cells by scavenging and engulfing cellular debris and pathogens (Lai and Todd, 2006).

The different numbers and functions of these cells are modulated by the local microenvironment and the interactions with surrounding cells (Bachiller et al., 2018).

Under physiological healthy conditions, microglial cells exist in a resting state, with small cell bodies and extensive processes, indicated as ramified state (Michell-Robinson et al., 2015). Resting microglia dynamically monitor the surrounding environment with their processes. When needed, in order to maintain CNS homeostasis, they perform pinocytose (Cherry et al., 2014; Prinz and Priller, 2014) and secrete growth factors for nourishing, supporting and protecting the electrophysiological functions of neurons (Eyo and Dailey, 2013). More, microglia monitor the synaptic function through synaptic contact, trimming or clearing damaged or redundant synapses (Zhang et al., 2020).

However, when their "sensors" bind to pathogen-derived molecules or to other insulting agents, the cells change into an activated state, modifying their morphology to an ameboid one and expressing inflammatory genes (Lai and Todd, 2006).

In addition, microglia have the ability to shift into different functional states, modifying their proliferation (Gomez-Nicola et al., 2013), phagocytic activity (Sierra et al., 2013), antigen presentation (Mcgeer et al., 1988b; Jimenez-Ferrer et al., 2017) and the release of inflammatory factors such as cytokines and chemokines (Kettenmann et al., 2011).

Microglial cells were first described by Franz Nissl in the late nineteenth century: with the name "Stabchenzellen" (= rod cells) he defined reactive glial elements with migratory, phagocytic and proliferative ability. After the identification of neuroglia by Virchow, in 1856, that corresponded to the macroglial population, composed by astrocytes and oligodendrocytes (Rio-Hortega, 1939), W. Ford Robertson described as mesoglia the mesoderm-derived phagocytic elements in the nervous system with a distinct origin from those of neurons and neuroglia. While this idea had merit, Robertson's mesoglia was found to correspond mainly to oligodendrocytes. Later, Santiago Ramon y Cajal renamed them as the "third element of the nervous system" to distinguish them from neurons and neuroglia, and supposed a mesodermal origin. After him, del Rio-Hortega, one of his students, refined the concept, identifying morphological and functional differences among the elements. In particular, he identified a non-neuronal, non-astrocytic third element, that he called microglial cell, distinct from neurectodermal oligodendroglia or oligodendrocytes (Rio-Hortega, 1939) (For historic review see Rezaie and Male, 2002). Even though the common believe at that time was a neuro-ectodermal origin of all the

glial cells, del Rio Ortega described them as mesodermal elements (Rio-Hortega, 1932; Ginhoux et al., 2013).

In fact, differently from astrocytes and oligodendrocytes, which shares their origin with neurons, microglial cells derived from the hematopoietic system during embryogenesis. Hematopoiesis can be divided in three fases:

- primitive hematopoiesis, which happens in the extraembryonic yolk sac (YS) and form nucleated erythroblasts and some macrophages,

- a 'transient' wave in which erythromyeloid progenitor cells (EMPs) migrate from the YS to the fetal liver,

- definitive hematopoiesis, in which hematopoietic stem cells (HSCs) give rise to all cell lineages (Dzierzak and Bigas, 2018).

Microglial cells are formed during the first fase, in the YS, at the onset of the second embryonic week in rodents. This was firstly hypothesized after the identification of F4/80+ cells in the developing brain before the onset of definitive hematopoiesis, and confirmed by genetic fate mapping, with the use of inducible Runx1Cre mouse lines to trace the trajectory of YS-derived cells into the brain (Alliot et al., 1999; Ginhoux et al., 2010; Stremmel et al., 2018).

Soon after birth, microglial cells acquire their definitive composition, both in numbers and phenotype. At early postnatal stages it has been reported a wave of microglial proliferation, but it is unclear if this could explain the numerical increase of microglial cells or indicate the possible contribution of blood derived monocytes (Alliot et al., 1999; Tambuyzer et al., 2009).

Once in the brain, they spread and diffuse in a non-heterogeneous manner throughout the CNS (Bachiller et al., 2018).

It is known that microglia are not replaced by HSC-derived cells; they are longlived, with an estimated turnover rate of 8–41 months in postnatal mice, depending on brain region (Lawson et al., 1992; Fuger et al., 2017; Tay et al., 2017); and with a prediction of 20 years in human (Askew et al., 2017; Reu et al., 2017). More, microglial cells are not replaced from myeloid cells of the bone marrow (DiSabato et al., 2016).

Although certain experimental conditions showed the postnatal infiltration of circulating monocytes and further differentiation to microglia, populating the corpus callosum (Ling

et al., 1980), or repopulating the whole microglial population (Beers et al., 2006), there is a lack of further quantitative data in the literature, that would be a key to understand the final composition of the microglial population. In addition, experiments that used transgenic tagging of E7.25- versus E8.5- derived cells strongly supported the idea that the microglial population resulted almost completely from the yolk sac, without the contribution of blood-derived monocytes (Ginhoux et al., 2010; Schulz et al., 2012; Kierdorf et al., 2013). This could be due to the selective nature of the blood-brain barrier (BBB), that corresponding studies have defined to be established in rodents at E13.5, in a period that precedes the release of monocytes into circulation and follows the invasion of yolk sac-derived cells (Daneman et al., 2010).

However, to date, the exact rates and regulation of microglial turnover in the healthy brain is still unclear. Lawson and others demonstrated that microglia cells can proliferate in the healthy brain, but they do more slowly that other tissue macrophages; they discovered that only the 0.05% of the microglia proliferated at a given time, that is 20 times less than the lowest labeling index for any other resident macrophage population studied (Lawson et al., 1992).

Evidence such as the discovery of Csf1r as an obligate microglial survival receptor (Dai et al., 2002) and the subsequent development of tools for microglia depletion suggested that microglial cells self-renew (Ajami et al., 2007), operating as their own progenitors (Bennett and Bennett, 2020).

However, the turnover rate in the absence of pharmacological or genetic depletion is still unclear, yet it appears to be relatively low (Ajami et al. 2007, Ginhoux et al. 2010).

Moreover, there are not detailed information about the mechanisms that regulate microglial proliferation in the steady state, or their apoptosis, in order to maintain a balanced population. Undoubtedly, the knowledge about microglial origin has increased significantly in the last years, but there is still much to do to completely understand microglial dynamics (Gomez-Nicola and Perry, 2015).

Interestingly, microglial cells are the only member of the immune system to permanently reside in the brain tissue itself, making their identity the product of a unique combination of origin and environmental exposures (Bennett and Bennett, 2020).

In fact, based on the specific anatomical structure or the activation profile, microglial cells have different morphological features (Kreutzberg, 1996; Gomez-Nicola and Perry,

2015), lysosome content (Majumdar et al., 2007), membrane composition (Button et al., 2014), electrophysiological activities (De Biase et al., 2017) and gene transcriptome profile (Chiu et al., 2013; Hickman et al., 2013).

- FUNCTIONS OF MICROGLIAL CELLS

As presented above, in the 1920s and 1930s, Pio del Rio-Hortega described for the first time microglial cells (Zhang et al., 2021).

These cells have been showed to possess an active and homeostatic functions in both the healthy CNS and in injuries.

A common belief, for many years, was for microglial cells to be quiescent in normal physiological conditions. However, different two-photon microscopy studies in living mice showed that microglial processes move and survey the local environment through the formation of random filopodia-like protrusions and extending and withdrawing of bulbous endings (Davalos et al., 2005; Nimmerjahn et al., 2005). The elevated motility allows the microglial processes to recognize the status of their immediate microenvironment, in order to endocytose nutrients and to clear debris and apoptotic cellular material (Napoli and Neumann, 2009).

So, it is possible to say that defining microglia behaviour with the terms “resting” or “quiescent” in the healthy brain is now obsolete, because it indicates a degree of inactivity that does not correctly represent the in vivo observations in which microglial cells use their motile processes to actively scan the microenvironment, to interact with synapses and with oligodendrocyte-derived myelin (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009; Fitzner et al., 2011; Paolicelli and others 2011; Schafer and others 2012; Kettenmann and others 2013) and to perform pinocytose (Cherry et al., 2014; Prinz and Priller, 2014). Further, they produce growth factors, necessary to nourish, support and protect the electrophysiological functions of neurons (Eyo and Dailey, 2013).

However, ischemia, infection and other factors can trigger microglial cells that, within minutes, proliferate, migrate to the diseased site, and change morphology, shortening their cell body protrusions and becoming round or ameboid. At this time, activated microglial cells are indicated as M1 type, that is the classic activated type, in which the cells assume a pro-inflammatory role (Zhang et al., 2021).

In this phase, the transcriptional profile is rapidly altered, leading to the production of pro-inflammatory cytokines and chemokines, ROS and NO (Zhou et al., 2006). In addition, active microglia go through cytoskeletal rearrangements that alter the pattern of the receptor expressed on the surface, allow the migration towards the injured or infected sites (Russo and McGavern 2015) and increase their phagocytic efficiency (Davalos et al. 2005). In the first phase, microglial activation has the function to protect the CNS and benefit the host organism (DiSabato et al., 2016).

Phagocytosis that is the removal of cellular debris by phagocytes, is a process that microglial cells have in common with macrophages. It consists in engulfing the cellular debris in phagosomes, membrane protrusions that merge with lysosomes and end with degradation, and is crucial for the preservation of tissue homeostasis throughout the lifespan. In particular, the phagocytosis of apoptotic cells is important in order to prevent the spill over of cellular contents that drives the migration of T cells, and to elicit anti-inflammatory responses (Savill et al., 2002; Gregory and Pound, 2011¸Tremblay et al., 2011)

However, recently data from both in vitro (Neher et al., 2011) and *in vivo* experiments (Fricker et al., 2012; Neher et al., 2013) demonstrated that microglial cells remove also the damaged but potentially viable neurons. This process, indicated with the term "phagoptosis", that describe the selective attack and removal of damaged but viable neurons by microglia, resembling similar cell-cell interactions observed in peripheral organs (Brown and Neher, 2012), is known to contribute to brain pathologies with a neuroinflammatory component and to increase the severity of the pathology it-self (Gomez-Nicola and Perry, 2015).

In the later stages of acute inflammation, microglial cells undergo another transformation into the M2 type, that is the anti-inflammatory one, responsible of resolving the inflammatory response through the secretion of anti-inflammatory cytokines and contributing to neuroprotection (Liu et al., 2019).

However, when the response mediated by the M1 type becomes amplified and exaggerated, leading to a chronic microglial activation and to a persistent neuroinflammation, the microglial role turns out to be detrimental and dangerous: it can lead to pathological changes and neurobehavioral complications such as depression and cognitive deficits (Norden and Godbout, 2013).

So, however microglial cells play an important role in the normal and healthy brain homeostasis, it is also known that these cells are pivotal in the coordinated communication between the immune system and the brain. In fact, in infection or disease, microglia cells are the first line of defence, becoming 'activated' and functioning as inflammatory cellular mediators (Zhang et al., 2021).

In particular, after an ischemic event, with a reduction in cerebral blood flow, microglial cells undergo morphological changes in preparation for the upcoming immune response (Masuda et al., 2011). In a word, microglia are associated with neuroinflammation, which plays an important role in ischemic stroke.

- ISCHEMIA AND MICROGLIAL CELLS

In healthy conditions, microglial cells have a down-regulated expression of molecules like CD45, Fc receptors, or MHC class II (Perry and Teeling, 2013). More, their interaction with astrocytes and neurons provides a regulatory system that keeps the inflammatory pathways of microglia under control. The expression of MHCII and its co-stimulatory molecules B7 and CD40 in microglia is inhibited by molecules like NGF or BNDF, respectively (Neumann et al., 1998; Wei and Jonakait, 1999) and neurotransmitters like GABA can modulate the inflammatory role of microglia (Pocock and Kettenmann, 2007). An important part in this system is played by cell–cell interactions. One example is represented by the CD200- CD200R systems of neurons, that, through the use of ITIM motifs (immunoreceptor tyrosine-based inhibitory motif), inhibits the inflammatory activation of microglia (Billadeau and Leibson, 2002).

Of course, when the control of these systems is lost, for example when neuronal degeneration occurs, an inflammatory reaction is triggered (Bhaskar et al., 2010; Zhang et al., 2011b). However, the idea that microglial cells need to be inhibited was driven by the initial views of these cells as "the bad guys"; instead, microglial cells functions and the maintenance of homeostasis are more complex.

It is known that in ischemic stroke, because of the activation of pro-inflammatory genes, the permeability of the blood–brain barrier (BBB) increases, leading to an infiltration of peripheral macrophages into the brain parenchyma (Lai and Todd, 2006). However, the first cells that react to cerebral ischemia are the resident brain microglia. In fact, in animal models of cerebral ischemia, it has been shown that, following vessel occlusion, the low

energy supply activates microglia within minutes, when any evident neuronal death has occurred yet (Nakajima and Kohsaka 2004).

After having received the signals, such as chemoattractive and activating ones from injured neurons or from the hypoxic event itself, microglial cells start to produce and release different effector molecules, such as the cytokines. In particular, a production of the pro-inflammatory type has been reported after the ischemic event. This post-ischemic period has been analysed significantly over the years; in fact, the neuronal dead that occur in this period of time seems to be largely caused by pro-inflammatory cytokines. Among them IL-1β and TNF-α are the two principal pro-inflammatory ones. Their overexpression has been shown to intensify ischemic damage, and their inhibition to reduce infarct volume (Yamasaki et al., 1995; Loddick and Rothwell, 1996; Barone et al., 1997).

Studies on ischemia models reported an upregulation of both IL-1β (Sairanen et al., 1997; Zhang et al., 1998; Davies et al., 1999; Mabuchi et al., 2000; Clausen et al., 2005) and TNF-α (Buttini et al., 1996; Botchkina et al., 1997; Uno et al., 1997; Gregersen et al., 2000; Dziewulska and Mossakowski, 2003) in post-ischemic microglia too.

This increase was also found in the levels of TNF-α receptors TNF-R1 (Botchkina et al., 1997; Sairanen et al., 2001; Dziewulska and Mossakowski, 2003) and TNFR2 (Botchkina et al., 1997; Dziewulska and Mossakowski, 2003) expressed by microglial cells after ischemia. On the contrary, the expression of IL-1R1, the receptor for IL-1β, in microglial cells is still not well documented, because this receptor is mostly expressed by neurons after an ischemic event (Sairanen et al., 1997).

In addition, different studies reported for these cytokines and their receptors to be very much dependent on the ischemia model and the animal strain used, suggesting that the system of their regulation has rather sensitive responses (Lai and Todd, 2006).

The excessive microglial activation and the subsequent secretion of pro-inflammatory mediators is associated also to the cerebral ischemia-reperfusion injury (Ekdahl et al., 2003; Xiong et al., 2016).

Numerous studies, in fact, have reported the role of the increased levels of pro-inflammatory cytokines, such as IL-6, IL-1β, IL-12, IL-23 and TNF-α, of inducible nitric oxide synthase and of ROS in perpetuating the inflammation and exacerbating brain damage (Xiong et al., 2016; Voet et al., 2018) through the activation of other

inflammatory cells, such as neutrophils. Chen and colleagues reported a role of M1 phenotypic microglia also in BBB destruction after ischemic stroke, imputing it to, among all, the increase of TNF-α (Chen et al., 2019).

The escalation in ROS production by microglial cells has been associated to the secondary cells death in penumbral areas that surround the ischemic core (Dirnagl et al. 1999; Danton and Dietrich 2003).

Therefore, these results suggest the importance of finding an effective therapy to block microglial-associated neuroinflammation in order to reduce BBB breakdown and diminish tissue damage after ischemic stroke (Zhang et al., 2021).

In fact, in spontaneously hypertensive rats with permanent middle cerebral artery occlusion (MCAO), a decreased infarct volume was reported after repetitive hyperbaric oxygen treatment, that was attributed to a suppressed microglial activation (Gunther et al. 2005).

Nowadays, different ways to modulate the microglial responses has been examined as possible therapeutic interventions for various neurological diseases (Garden and Moller, 2006). One option is represented by specific viral vectors, that can be used to transport effective targeted gene to microglial cells (Balcaitis et al., 2005); however, their effectiveness *in vivo* requires more investigations. Several groups reported that active or passive immunization with myelin-derived proteins, modulating the systemic immune system, was able to decrease infarct volume and mitigate the neuroinflammatory response resulting after acute ischemic injury (Becker et al.,1997; Frenkel et al., 2003). Interestingly, in this model, the administration of the lipopolysaccharide (LPS) (a component of the outer membrane of Gram-negative bacteria that triggers microglial cells) during reperfusion time led to the increase in the pro-inflammatory responses and the neurological damage (Becker et al., 2005). The mechanisms behind these answers are still not completely understood. Another option is represented by pharmacologic agents that can modulate microglial functions (by both mitigation of the pro-inflammatory pathways and increasing of alternative immunomodulatory programs). One example is minocycline that possesses anti-inflammatory properties due to the downregulation of microglial activation. This drug was administrated for 5 days starting within 6–24 h of stroke onset in one small randomized, controlled, clinical trial. After 90 days, significantly better neurological outcomes were reported in the minocycline-treated patients (Lampl et al., 2007). However, there is still the need of larger randomized clinical

trials to determine if minocycline is an effective treatment for acute ischemic stroke and of additional basic science mechanistic studies to investigate the contribution of microglial inhibition to any potential therapeutic effect, considering also that minocycline has numerous mechanisms of action. Nowadays, an increasing interest in different molecular targets, that are expressed mostly in microglial cells (even if not exclusively) is emerging. Among these there are TLRs, thrombin receptor (proteolytically activated receptor [PAR]1), and prostaglandin receptor antagonists, that are being evaluating as potential therapeutic targets in acute ischemic stroke in humans. Great attention has been given to the ability of these antagonists to attenuate negative inflammatory responses in the penumbra following stroke, in order to be used as treatments soon after the stroke (Weinstein, 2010).

However, to date, there are still not available therapeutic strategies that specifically target microglia, indicating PEMFs treatment as a valid and safe option.

CYTOKINES: THE PRINCIPAL MEDIATORS REGULATED BY MICROGLIA

The term “cytokines” indicates the group of soluble extracellular proteins and glycoproteins, formed in response to injurious stimuli by every nucleated cell and that differs from hormones, that are produced in clusters of cells organized into glands. Usually, these molecules act locally, interacting with cells expressing complementary receptors. They control the interaction of immune cells, such as T cells, B cells and monocytes/macrophages and have a crucial role in the immune response (Arai et al., 1990). Their role in the NS, where they have been primarily involved in the pathogenesis of autoimmune diseases such as human multiple sclerosis (Merril and Benveniste, 1996), is well known. However, cytokines exert various actions in the brain; in fact, they modulate the systemic host responses to diseases and the local changes caused by CNS infection, injury and inflammation (Rothwell and Hopkins, 1995; Rothwell et al., 1996). This large group includes different polypeptides: the interleukins, chemokines, tumor necrosis factors, interferons, growth cell stimulating factors and neurotrophins. Cytokines play different roles on various areas such as cell growth and differentiation, immune and

inflammatory responses, and on different physiological systems in both healthy and ill conditions. In the healthy adult brain, most cytokines are expressed at low or undetectable levels; however, in response to injury or infection, their concentration increases, due to their induction (Benveniste et al., 1990; Aloisi et al., 1992). One example is represented by the IL-1, 2, 3, 4, 6, 8 and 10, several chemokines, TNF, interferons and numerous growth factors, whose expression is induced rapidly by experimental and clinical insults to the CNS. The different roles of many of these cytokines in the brain are still not clear, but probably they are associated to both beneficial and harmful effects. Among them all, the three major cytokines produced by cultured brain cells after various stimuli are TNF-α, IL- 1β, and IL-6.

- **Origin, functions and role in cerebral ischemia of the pro-inflammatory cytokine TNF-α**

The fact that bacterial-derived endotoxins were able to provoke, in humans, hemorrhagic necrosis of tumors, that consists in cell swelling, organelle destruction, and finally cell lysis (Chu, 2013) was known; however, until 1962 it was not suspected that this effect was indirect. In fact, O'Malley et al. (O'Malley et al., 1962) first demonstrated that animals not exposed to LPS but to serum from animals treated with LPS could develop hemorrhagic necrosis of tumors, suggesting that this effect was mediated by a "tumor-necrotizing factor". One decade later, Carswell and colleagues (Carswell et al., 1975) identified the substance that they named "tumor necrosis factor" (TNF) and demonstrated that it can cause necrosis of several transplanted tumors (Parameswaran and Patial, 2010).

Subsequent investigations discovered that this pro-inflammatory cytokine exerts different roles. Studies in TNF-α- or TNF receptor (R)-deficient mice found an important role of TNF-α in the regulation of embryo development and in the sleep-wake cycle. More, TNF was shown to be essential for lymph node follicle and germinal centre formation, and also in host defence against bacterial and viral infection. Among its functions, TNF was found to be an endogenous pyrogen that causes fever, and chronic exposure to this cytokine was reported to cause cachexia, wasting syndrome, and depression (Chu, 2013).

TNF showed to play a crucial role in animal models of endotoxin-induced septic shock and in chemotherapy-induced septic shock in late-stage lung cancer patients, but also in autoimmune diseases such as rheumatoid arthritis (RA), inflammatory bowel diseases including Crohn's disease and ulcerative colitis, multiple sclerosis, systemic lupus erythematosus and systemic sclerosis. In addition, TNF was reported to be an important risk factor in case of tumorigenesis, tumor progression, invasion and metastasis and to be involved in cancer-associated chronic inflammation (Fig. 13). In fact, the longterm usage of aspirin was able to decrease TNF secretion leading to a significant reduction of the incidence of human colorectal colon cancer. Furthermore, research on TNF levels in biopsy samples from human breast, ovarian and renal cancers, and in adjacent stromal cells reported its presence (Carbone et al., 2012). In experimental murine cancer models, the pro-inflammatory cytokine was reported to be involved in the promotion of early-stage liver and skin cancer, and in the enhancement of colon cancer metastases to the lung (Chu, 2013).

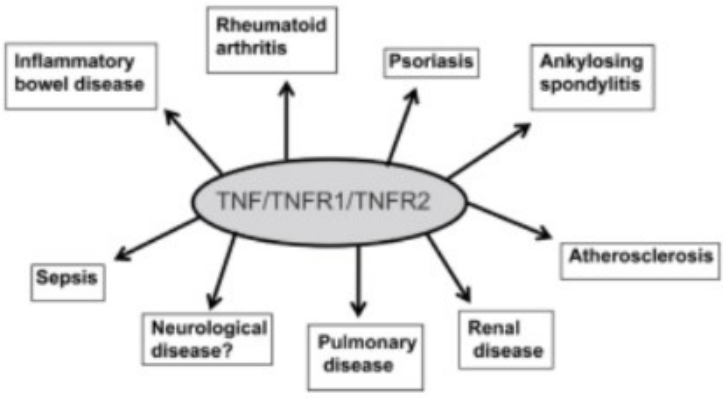

Figure 13. TNF-α and its receptors play a major role in a number of inflammatory diseases. Anti-TNF-α agents are clinically used to treat some of these diseases (Parameswaran and Patial, 2010).

TNF is a key mediator of both acute and chronic systematic inflammatory reactions; it is known its ability to control many aspects of macrophage function. In fact, TNF is secreted soon after a possible deleterious event, such as trauma, infection, or exposure to bacterial-derived LPS, and in inflamed tissue (Feldmann et al., 1994). Among its functions, a fundamental one is to regulate the other pro-inflammatory cytokines, orchestrating their cascade (Maini et al., 1995). At the same time, it also regulates pro-inflammatory cells activation, operating on the increase of lipid signal transduction mediators such as prostaglandins and platelet activating factor (Vassalli, 1992). Because of that, it has been

implicated in the development of many chronic inflammatory diseases (Clark, 2007) and in the development of cardiovascular disease in these subjects. In fact, the TNF-α blockade in rheumatoid arthritis patients showed to reduce the cardiovascular risk (Wolfe and Michaud, 2004).

In TNF-α stimulated macrophages, it has been found a production of TNF-α, induced by its own secretion through toll-like receptors stimulation, and an activation of the cells themselves. However, when the TNF-α is administered exogenously, macrophages activation is mediated by interferon gamma (IFNγ). The two molecules activate macrophages: TNF-α induces the activation of NFκB, while IFNγ induces the nuclear localization of STAT1α, precluding its recruitment from TNFR1 and enhancing the TNFα-induced NFκB activation (Wesemann and Benveniste, 2003). Once triggered, macrophages migrate to the inflammatory sites, where they lyse the pathogens thanks to the increased production of ROS and the induction of iNOS, that leads to NO production.

In the CNS, TNF-α exerts a considerable role in the pathogenesis of neurological diseases. In fact, microglial cells, which are the first line of defence of the brain, are a significant producer of this cytokine (Feuerstein et al., 1994).

TNF-α seems to play a role in blood-brain barrier, inflammatory, thrombogenic, and vascular changes associated with brain injury (Feuerstein et al., 1994; Arvin et al., 1996; Kochanek and Hallenbeck, 1992). In fact, its level was found to be increased in tissue, cerebrospinal fluid and plasma of patients affected by CNS disorders, such as Alzheimer's disease (Fillit et al., 1991) multiple sclerosis (Sharief et al., 1993) Parkinson's disease (Mogi et al., 1994) and meningococcal meningitis (Waage et al., 1989). Different cell types have been recognised as TNF-α producers following various kinds of stimulations/injuries; among these the ependymal cells of the choroid plexus (Tarlow et al., 1993), astrocytes and microglia (Chung and Benveniste, 1990), microglia and macrophages after ischemia (Liberman et al., 1989; Ganter et al., 1992) and central neurons after cerebral focal ischemia (Buttini et al., 1994).

Connected with the TNF-α increase, p55 and p75 receptors (that are TNF receptors) also resulted upregulated within 6 and 24 hr, respectively, from the ischemic event. The association between the upregulation of both p55 and the pro-inflammatory cytokine suggest a deleterious role of the increased TNF- α production during the acute response to cerebral ischemia, considering that the p55 receptor has been associated to a cytotoxic signaling.

Studies in transient occlusion of the middle cerebral artery (MCAO) showed an increase in TNF-α level, that return to basal after two days (Liberman et al., 1989). Its negative role appeared when injected, in an intra-cerebro-ventricular way, 24h before the occlusion, causing an increased infarction size that was reversed with the administration of anti-TNF antibodies (Wang et al., 1994a). This evidence suggested that an augmented TNF-α level could lay the ground for subsequent damage, leading to the switch to a pro-adhesive state in capillary endothelium, and to the apoptosis of different cells (Selmaj et al., 1990; Fehsel et al., 1991; Wang et al., 1994b).

Further studies suggested a role of TNF-α in all the stroke-associated aspect. It was reported to increase the tissue factor and adhesion molecules in leukocytes, but also the production of IL-1, NO, factor VIII/von Willebrand factor, platelet activating factor endothelin and plasminogen activator inhibitor-1, and to reduce tissue plasminogen activator. More, it acts also on vascular permeability, through the activation of matrix metalloproteinases. This pro-inflammatory cytokine stimulates the pro-inflammatory and pro-coagulant function of endothelium (Pober and Cotran, 1990) and regulates the induction of numerous enzymes, such as xanthine oxidase, cyclooxygenase (COX) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase, in order to increase the ROS production (Terry et al., 1999; Weber et al., 1994). All these provoke thrombosis, haemorrhage and predispose to local inflammation, creating a condition which may be essential in initiating the stroke and in increasing the stroke-related brain damage (Tuttolomondo et al., 2008).

A role of TNF-α has been also described in neuronal death. In fact, a study on HT22 hippocampal cells and on mouse primary cortical neurons, exposed to the TNF-α level seen in blood following stroke (up to 1000 pg/ml), reported a decrease in mitochondrial oxygen consumption rates and in membrane potential, and a decrease in ATP- production oxygen related consumption. The authors suggested that these were signals of an imminent cell death (Wilkins and Swerdlow, 2015).

More, the TNF-α produced by microglial cells can cause the necroptosis of endothelial cells, that is a fundamental step for the BBB collapse after I/R injury.

Because of the evidence above, targeting inflammation through TNF-α seems to be effective to protect the BBB and CNS cells from death (Chen et al., 2019).

- **Origin, functions and role in cerebral ischemia of the pro-inflammatory cytokine IL-6**

IL-6 is a soluble pyrogen that exerts a variety of effects on inflammation, immune response, and hematopoiesis. However, its name has a recent origin: only in 1989 Kishimoto found out that different molecules, named with their functions, were the same mediator (Kishimoto, 1989). Among them the name B-cell stimulatory factor 2 (BSF-2), hepatocyte-stimulating factor (HSF), hybridoma growth factor (HGF) and interferon (IFN)-β2, which referred to its ability to differentiate activated B cells into antibody (Ab)-producing cells (Kishimoto, 1985), to stimulate the protein synthesis on hepatocytes, to enhance the growth of hybrid cells (plasma cells and myeloma cells) and for its IFN antiviral activity, respectively (Tanaka et al., 2014).

To exerts its effects, this cytokine binds to a specific receptor, IL-6 R, that activate various signaling pathways leading to gene activation (Van Wagoner and Benveniste, 1999). It is reported that IL-6 levels in healthy conditions is low, while it increases during CNS disorders. The production of this mediator is induced by a variety of molecules, such as IL-1, the transforming growth factor-β and prostaglandins (PGs), and others that act indirectly, strengthening them and increasing IL-6 such as β-amyloid, interferon-γ (IFN γ) and IL-4. IL-2, instead, can act both directly and indirectly through the increase of IL-1β and TNF-α levels (Tuttolomondo et al., 2008).

IL-6 exerts an important inflammatory role; in fact, it belongs to that group of cytokines that is overproduced during inflammation, and that stimulate the production of other pro-inflammatory molecules. Like IL-1β and TNF-α (Kushner, 1993), it is produced by monocytes and macrophages in the inflammatory sites.

IL-6 takes part in the acute phase reactions, but it is also involved in the phase of construction of specific cellular and humoral immune responses, such as in the end-stage B cell differentiation, in the T cell activation, in the immunoglobulin secretion and in the transition between acute and chronic inflammation (Kaplanski et al., 2003) (Fig. 14).

Numerous studies reported a role of IL-6 in cerebral ischemia. In fact, it is known the role of inflammation in acute cerebral ischemia and that this is mediated by the increase of certain cytokines. Among these, an increased level of IL-6 was reported, in patients, in

both the CNS and the systemic circulation (Emsley et al., 2003; Lakhan et al., 2009; Denes et al., 2010; Bharosay et al., 2011; Hopkins et al., 2012). After the ischemic event, this cytokine acts as a messenger between the vascular endothelium, leucocytes, and parenchyma resident cells (Shaafi et al., 2014).

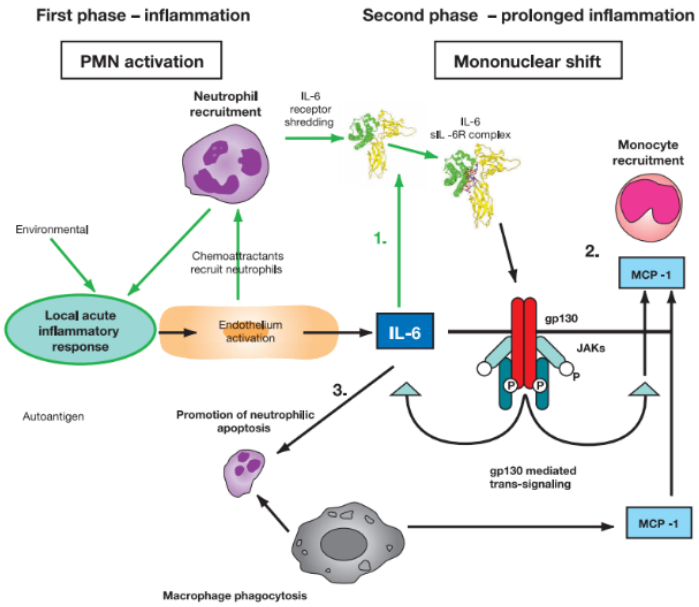

Figure 14. Possible role played by IL-6 in the shift from acute to chronic inflammation. Stage 1: following acute inflammatory response, IL-6 can bind with sIL-6R. Stage 2: trans-signaling through gp130 leads to monocyte recruitment. Stage 3: prolonged IL-6 leads to neutrophilic apoptosis, phagocytosis and mononuclear accumulation at the site of injury. IL, interleukin; JAK, Janus activated kinase; MCP, monocyte chemoattractant protein; PMN, polymorphonuclear neutrophil; sIL-6R, soluble IL-6 receptor (Gabay, 2006).

Because of the fact that the role of inflammatory cytokines in the early neurological deterioration and in the infarct volume are known (Rodriguez-Yanez and Castillo, 2008), different studies investigated the possible association between the level of IL-6 and the ischemic stroke outcome.

Shaafi and colleagues demonstrated the link between the increased IL-6 level and the severity of neurological damage (both in clinical and imaging aspects) and of prognosis, in patients with acute ischemic stroke (Shaafi et al., 2014). More, the investigation of IL-6 levels in patients with stroke, on admission and 28 days after the onset, reported a possible association between the cytokine and both the severity of lesion and the outcome of patients (Nakase et al., 2008). A confirmation was reported by the assessment with the Barthel index of 1-month outcome of stroke, that showed that both IL-6 and nitrate levels

measured 6 hours after the ischemic event were related to the outcome (Beridze et al., 2011), such as the level of the combination among the cytokine and heart type fatty acid binding protein (Park et al., 2013). Other studies identified the increased plasma levels of the cytokine as a predictor of a poor outcome more in elderly people than younger (Shakarishvili and Beridze, 2008) and the plasma levels of IL-6 (and also fibrinogen, serum albumin and white blood cells) as a predictor of 6-month stroke recovery (Clark et al., 1998).

The identification of IL-6 as a possible biomarker for the risk of 12-month mortality was assessed by Shenhar-Tsarfaty and colleagues, that detected a cut point (6.47 pg/ml) for patients at a high risk (Shenhar-Tsarfaty et al., 2010). The results was confirmed by Shaafi and colleague, that found a parallelism between the hight level of IL-6 and both mid-term outcome and the mortality rate (Shaafi et al., 2014).

- **Origin, functions and role in cerebral ischemia of the pro-inflammatory cytokine IL-1β**

IL-1, as suggested by the name it-self, is the first cytokine identified. Different actions and pathways are associated to this molecule; however, the purification of the protein was guided by the intention to investigate the factor responsible of inducing fever (or the endogenous pyrogen activity) (Dinarello, 1981; Auron et al., 1984). After that, thanks to the cloning of IL-1 cDNAs, two different isoforms were identified: IL-1α and IL-1β (Dinarello, 2011).

In detail, the purification of human IL-1β was performed in 1977, when it was reported to raise the fever in rabbits at 10 ng/kg (Dinarello et al., 1977). This characteristic was then confirmed in 1984, with the human cDNA clonation (Auron et al., 1984). Thanks to the IL-1β availability, multiple properties of this molecule were found. Among them, its important role in inflammation, which was confirmed inactivating the cytokine with specific monoclonal antibody in clinical trials (Dinarello and van der Meer, 2013). Similar experiments indicated a pivotal role of IL-1β in the pathogenesis of atherosclerosis (Ridker et al., 2017a) and in the progression of cancer (Ridker et al., 2017b).

Despite its low level in a healthy brain, IL-1β exerts important homeostatic functions: among them the control of sleep, feeding and temperature (Dinarello, 1996). On the contrary, the increased amount of IL-1β has been found in numerous diseases and its release is associated to different cell types.

IL-1β can be cleaved and activated by Caspase 1, via the inflammasome, which is involved in the innate immunity pathway and in inflammatory disorders (Martinon and Tschopp, 2007).

It is known that the inflammasome is implicated in different auto-inflammatory diseases; in fact, multiple evidence reported a mutation in the genes that encodes for it, with, as a result, an increase in IL-1β production (Stojanov and Kastner, 2005; Church et al., 2008). One example is represented by periodic-fever syndrome; a small clinical trial, in fact, reported an improvement of clinical symptoms when treated with IL1- receptor antagonist (IL1ra) that antagonise the effects of IL-1β (Hawkins et al., 2004; Hoffman et al., 2004).

In peripheral tissue, IL-1β showed to induce potent mechanical and thermal hyperalgesia (Fukuoka et al., 1994; Watkins et al., 1994; Safieh-Garabedian et al., 1995; Ferreira et al., 1988; Cunha et al., 2000; Zelenka et al. 2005); in the NS, it is involved in the development and maintenance of neuropathic pain (Scholz and Woolf, 2007; Uceyler and Sommer, 2008) and it is over-expressed in degenerate and herniated human intervertebral discs (LeMaitre and Hoyland, 2007).

This cytokine exerts an important role also in the CNS, where it seems to cause hyperalgesia and enhanced neuronal responses (Oka et al., 1993; Oka et al., 1994; Watkins et al., 1994; Reeve et al., 2000), and to be involved in the inflammatory status associated to different pathologies such as Alzheimer's disease and ischemic stroke.

The production of IL-1β has been associated to the activation of glia: in bone cancer pain and in intracerebral hemorrhage, astrocytes are important producers (Zhang et al., 2005; Wasserman et al., 2007) but also neurons and microglial cells activate a rapid secretion in numerous circumstances (Van Dam et al., 1995; Clark et al., 2006).

In fact, IL-1β increase has been connected to all the different levels of the inflammation, being involved in the activation of many cells of the innate immunity and in the induction of the cyclooxygenase-2 (COX-2), of iNOS, increasing the NO level, of many cytokines (including itself), of leukocyte adhesion molecules and thrombogenic mediators (Libby, 2017).

IL-1β was also reported to be increased in experimental brain damage (Beneviste, 1992; Toulmond et al., 1993) and to be involved in neuronal death (Tuttolomondo et al., 2008).

Numerous evidence present IL-1β as an important mediator in the evolution of the infarct (Rothwell and Relton, 1993; Rothwell and Strijbos, 1995; Rothwell et al., 1997; Loddick et al., 1998; Rothwell and Luheshi, 2000; Fogal and Hewett, 2008); in fact the administration of both antibody against the cytokine or the IL1 R, was reported to significantly decrease the subsequent cerebral ischemic damage (Relton and Rothwell, 1992; Betz et al., 1995; Loddick and Rothwell 1996; Yang et al., 1997; Yang et al., 1998; Yang et al 1999; Mulcahy et al., 2003). The lack of ICE, an enzyme involved in the IL-1β activation, in animals deficient of it, showed a reduced infarct volume in parallel with a decreased cytokine level (Hewett et al., 2012) and transgenic mice with mutant ICE gene were reported to develop smaller infarcts, to have less neurological deficit, a decreased IL-1 level and a reduced DNA fragmentation after both transient and permanent MCAO (Loddick et al., 1996; Friedlander et al., 1997).

In addition, the lack in the cytokine or in its receptor makes brain injury size reduced after MCAO if compared with wild-type (Boutin et al., 2001; Ohtaki et al., 2003; Fogal et al., 2007). In the same direction there is the Yamasaki et al. work, that demonstrated that the injection of IL-1β provokes neuronal injury in MCAO rats (Yamasaki et al., 1995), and the finding of an increased IL-1β concentration in the cerebrospinal fluid of stroke patients (Tarkowski et al., 1999; Gusev and Skvortsova, 2003). Because of these data, a trial with recombinant human (rh)IL-1ra in patients with acute stroke was made and published with positive results (Emsley et al., 2005), confirming the importance of decreasing the pro-inflammatory cytokine IL-1β in order to improve the recovery from cerebral ischemia (Hewett et al., 2012).

AIM OF THE STUDY

Stroke is a leading cause of mortality and disability worldwide, with a high incidence and a dramatic public health burden. In this condition, a fundamental role is played by neuroinflammation, that triggers the immune system cells, worsening the lesion (Lehmann et al., 2014; Arnao et al., 2016; Bustamante et al., 2016;).

To date, the only available therapeutic approaches are represented by cerebral artery recanalization and antithrombotic therapies, that unfortunately have a restricted time window and a possible negative outcome such as the disruption of blood-brain barrier, hemorrhagia and brain edema (Molina et al., 2009).

An opportunity in this field is represented by PEMFs, that have showed to possess anti-inflammatory capacity and to exert beneficial effects in numerous inflammatory disorders including pseudoartrosis, joint disease, complications related to diabetes mellitus, impaired wound healing, pain and neurodegenerative pathologies (Roland et al., 2000; Canedo-Dorantes et al., 2002; Heden and Pilla, 2008; Weintraub et al., 2009; Arendash et al., 2010; Cebrian et al., 2010; Goudarzi et al., 2010; Jing et al., 2011; Pan et al., 2013; Varani et al., 2012, 2017; Iwasa and Reddi, 2018).

Recently, PEMFs have been found to reduce the release of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in LPS-activated N9 microglial cells, suggesting that PEMFs could represent a potential therapeutic approach in cerebral ischemic conditions (Vincenzi et al., 2017). However, how this occurs is still unknown and the intracellular pathways activated by PEMFs to trigger anti-inflammatory effects have not been investigated, making necessary to shed light on the cellular molecules implicated in their protective effect. In particular, the signaling involving 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) and Akt, in activated N9 microglial cells, was the focus of this study. In addition, PEMFs effects on ROS as well as on microglia invasion and phagocytosis provoked by LPS insult were addressed.

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 pJNK1/3(pThr183/Tyr185) assay kit was from PerkinElmer (Milan, Italy). PKC-ε translocation inhibitor peptide was purchased by Calbiochem (Milan, Italy). TNF-α and IL-1β AlphaLISA kits were obtained from Perkin-Elmer (Milan, Italy). IL-6 Quantikine ELISA kit was purchased from R&D Systems (Milan, Italy). Unless otherwise noted, all other reagents were purchased from Sigma (Milan, Italy).

CELL CULTURES

The N9 murine microglial cell line was cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% heat inactivated australian fetal bovine serum, glutamine, 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, cell culture medium was replaced with fresh serum-free medium for another 24 h, to minimize the interference of growth factors present in the serum with the signal transduction.

ELECTROMAGNETIC FIELD TREATMENT

N9 cells were treated with PEMFs produced by a complete generator system (IGEA, Carpi, Italy) that has been exhaustively described and used elsewhere (Cadossi et al., 1992; Massot et al., 2000; Barbault et al., 2009; De Mattei et al., 2009; Ongaro et al., 2012; Varani et al., 2002, 2003, 2008, 2012; Massari and Cadossi, 2013; Vincenzi et al.,

2013). PEMFs characteristics were the following: 1.3 ms pulse duration and 75 Hz frequency, resulting in a 0.1 duty cycle. The magnetic field generated presented a peak intensity value of 1.5 ± 0.2 mT as measured by the Hall probe (HTD61-0608-05-T, F.W. Bell, Sypris Solutions, Louisville, KY) of a gaussmeter (DG500, Laboratorio Elettrofisico, Milan, Italy) presenting 0.2% of reading sensitivity. The electric field obtained showed a voltage of 2.0 ± 0.5 mV peak amplitude evaluated through a standard coil probe. A digital oscilloscope was used to evaluate the temporal pattern of the signal (Le Croy, Chestnut Ridge, NY). The shape and impulse length of the produced electric voltage was kept unchanged.

CELLULAR TREATMENTS

LPS, a cell wall component of Gram-negative bacteria, is a potent activator of glia. Hence, N9 microglial cells were treated with 1 μg/ml LPS for different times (from Escherichia coli, serotype 055:B5, soluble in cell culture medium) with and without PEMFs. Microglial cells were incubated with signaling inhibitors (1 μM SQ22,536, U73122, PKC-ε translocation inhibitor peptide, Rottlerin, U0126, SB202190, SP600125, SH5 and 10 μM casp 1, inhibitors of AC, PLC, PKC-ε, PKC-δ, ERK1/2, p38, JNK1/2, Akt, Caspase 1, respectively) for 30 min prior to LPS treatment, then maintained in IMDM and harvested after treatment at the indicated times. Inhibitors of kinases, and inhibitor of TLR4 receptor CLI-095 were made up in dimethyl sulfoxide solution (DMSO) and then diluted in cell culture medium (0.1%–0.2% of DMSO). An equal amount of DMSO was used in control.

ALPHALISA

The levels of TNF-α and IL-1β proteins secreted by the cells in the medium were determined by AlphaLISA kits (Perkin Elmer, Milan, Italy). Cells were incubated in 24 well at 37 °C for 24 hr. Then, 5 μl of cell conditioned medium containing the secreted cytokines were transferred in the assay white plates with 10 μl of the mixture containing Acceptor beads conjugated with anti-TNF-α or -IL-1β antibodies. After 30 min, 10 μl of the biotinylated antibody anti-analyte were added for 1 hr and then 25 μl of 2X SA-Donor beads solution were supplemented. The plate was incubated, in dark conditions, for 30 min and finally read in the AlphaScreen mode on the Ensight plate reader (Perkin Elmer).

After excitation by laser at 680 nm, the singlet oxygen released by alpha Donor bead travels to the nearby Acceptor bead that emits fluorescence at 615 nm. The increase of AlphaLISA signal was proportional to the concentrations of TNF-α or -IL-1β.

ELISA ASSAY

The levels of IL-6 protein secreted in the medium were determined by mouse Quantikine ELISA kit (R&D Systems, Milan, Italy). In brief, sub-confluent microglia, 20,000 cells in 24-well dishes, were plated and allowed to attach overnight. The following day the medium was changed into a fresh one and solvent or various concentrations of drugs were added. After the incubation time, the medium was collected, and IL-6 protein concentrations were measured by ELISA according to the manufacturer's instructions.

ALPHASCREEN SUREFIRE PJNK MAPK TEST

AlphaScreen SureFire phospho(p) JNK1/2 (pThr183/Tyr185) assay kit (Perkin Elmer, Milan, Italy) was used. N9 microglial cells were plated in IMDM medium into 96-well plates, and then maintained at 37 °C, for various time points. Cells were activated with LPS, lysed and then beads associated to anti-p-JNK- and anti-JNK-antibodies were added, as described in the protocol provided with the kit. At the end, the Ensight Perkin Elmer-multimode plate reader (Perkin Elmer, Milan, Italy) was used to reveal fluorescence at 615 nm.

MTS TEST

The MTS assay was carried out to evaluate proliferation of N9 cells by using the CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Milan, Italy). Briefly, cells were seeded in 24-multiwell plates, and exposed to PEMFs for 72 hr before addition of MTS solution. The reading of optical density was performed with a spectrophotometer at 570 nm.

ROS ASSAY

The ROS production was evaluated with a dihydroethidium (DHE) cellular ROS detection assay (Cayman Chemical, San Diego, CA). Cells were seeded in a black 96-well plate and after stimulation with LPS for 24, 48 and 72 hr in the absence or in the presence of PEMFs, a solution of DHE was added. Fluorescence was measured in a EnSight Multimode Plate Reader (Perkin Elmer, Boston, MA) with an excitation at 485 nm and an emission at 535 nm.

CELL INVASION ASSAY

Microglial cells were seeded in a 96-well plate in serum free medium and stimulated with LPS for 6 hr in the absence or in the presence of PEMFs. Cell invasion was quantified using a Cell invasion assay, according to manufacturer's instructions (Trevigen, Gaithersburg, MD). In particoular, the cells that passed through a specific layer were identified using a cell dissociation solution/Calcein-AM. The emitted fluorescence was measured in a EnSight Multimode Plate Reader (Perkin Elmer, Boston, MA) with an excitation at 485 nm and an emission at 520 nm.

PHAGOCYTOSIS ASSAY

Microglial cells were seeded in a 96-well plate and stimulated with LPS in serum free medium for 4 hr in the absence or in the presence of PEMFs. Phagocytosis was quantified by using a Phagocytosis assay kit, after addition of a green zymosan solution, according to manufacturer's instructions (Trevigen, Gaithersburg, MD). After a series of centrifuges, fluorescence was measured in a EnSight Multimode Plate Reader (Perkin Elmer, Boston, MA) with an excitation at 490 nm and an emission at 520 nm.

STATISTICAL ANALYSIS

Analysis of all data reported in the figures and text shows the mean ± standard error (SEM) of four different experiments. Statistical analysis was performed through one-way analysis of variance (ANOVA) and whether necessary Dunnett's test. A P-value less than 0.05 was evaluated significant from a statistical point of view.

RESULTS

Signaling pathways involved in PEMFs-induced reduction of pro-inflammatory TNF-α in N9 microglial cells

LPS stimulation (1 μg/ml) was able to activate N9 microglial cells mediating a significant increase of the pro-inflammatory cytokine TNF-α after 24 hr of incubation. Interestingly, PEMF exposure was able to significantly reduce the LPS-stimulated TNF-α production, thus inducing a protective effect. The involvement of AC, PLC, PKC-ε and PKC-δ in the TNF-α increase due to LPS activation and the molecules modulated by PEMFs to reduce this effect were investigated. N9 microglial 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 LPS for 24 hr, in the absence and in the presence of PEMFs. The effect of LPS on TNF-α production was not affected by inhibitors of AC, PLC, PKC-ε and PKC-δ, suggesting that these signaling pathways are not involved in LPS-induced TNF-α production. None of these inhibitors was able to revert PEMF-induced protective effect (Fig. 15). Furthermore, to examine whether ERK1/2, p38, JNK1/2 MAPK kinases, Akt and caspase 1 pathways were involved in the stimulatory effect mediated by LPS on TNF-α secretion, N9 were pretreated for 30 min with 1 μM U0126, SB202190, SP600125, SH5 and 10 μM casp 1, inhibitors of ERK1/2, p38, JNK1/2 MAPK kinases, Akt and caspase 1, respectively, before incubation with LPS for 24 hr, in the absence and in the presence of PEMFs. The effect of LPS on TNF-α production was not affected by inhibitors of JNK1/2 and Akt, whilst was reduced by U0126, SB202190 and caspase 1 inhibitor, suggesting that ERK1/2, p38 and caspase 1 are involved in LPS-induced TNF-α production (Fig. 16). However, SP600125 was able to revert the protective effect mediated by PEMFs, suggesting the recruitment of JNK1/2 MAPK kinases in this response. Finally, the effect of CLI-095, inhibitor of TLR4 receptor, was evaluated. This blocker completely reverted (95% ± 7 inhibition) the LPS-induced TNF-α stimulation, suggesting the involvement of TLR4 receptor in the LPS effect, but PEMF ability to reduce the TNF-α production (42% ± 5 inhibition without CLI-095) did not involve this receptor (43% ± 4 inhibition).

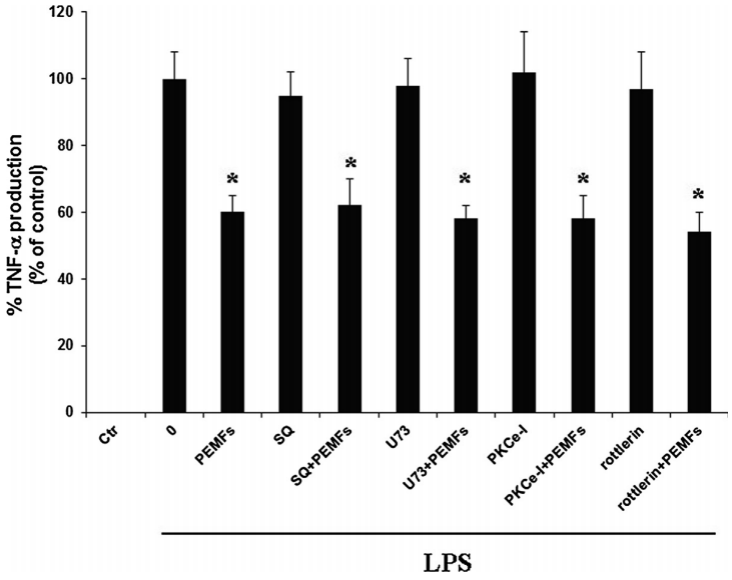

Figure 15. Effect of inhibitors of AC, PLC, PKC-ε, PKC-ε on LPS-induced TNF-α production from N9 microglial cells, in the absence/presence of PEMFs. N9 cells were incubated with 1 μM SQ22,536, U73122, PKC-ε blocker and rottlerin for 30 min before stimulation with LPS, in the absence/presence of PEMFs, for 24 hr. TNF-α production was evaluated by alphaLISA. *P < 0.01 versus 0 (LPS alone). Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA followed by Dunnett's test.

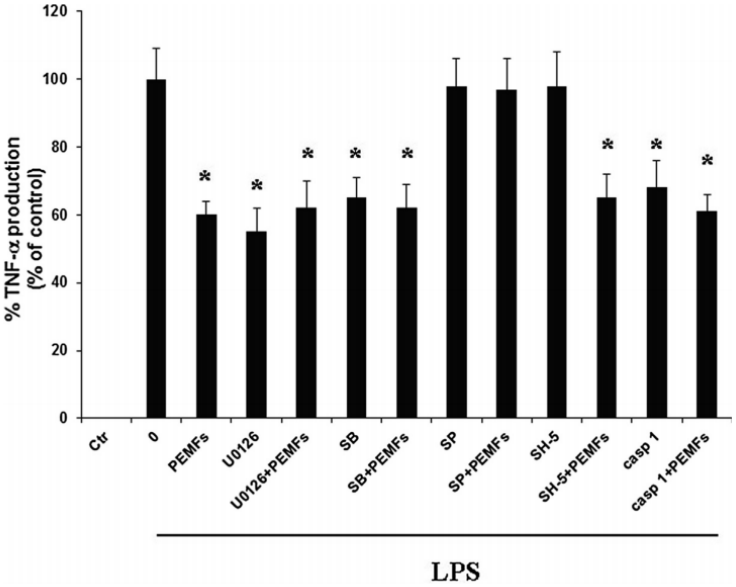

Figure 16. Effect of inhibitors of ERK1/2, p38, JNK1/2, Akt and caspase 1 on LPS-induced TNF-α production from N9 microglial cells, in the absence/presence of PEMFs. N9 cells were incubated with 1 μM U0126, SB202190, SP600125, SH5 and 10 μM caspase 1 for 30 min before stimulation with LPS, in the absence/presence of PEMFs, for 24 hr. TNF-α production was evaluated by alphaLISA. *P < 0.01 versus 0 (LPS alone). Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA followed by Dunnett's test.

Signaling pathways involved in PEMFs-induced reduction of pro-inflammatory IL-1β in N9 microglial cells

The effect of LPS on IL-1β modulation in N9 microglial cells was investigated. To reach a massive IL-1β release, N9 microglia was stimulated with ATP 5 mM for 30 min, before the assay. In this condition LPS strongly stimulated IL-1β secretion and PEMFs significantly reduced cytokine levels. The involvement of AC, PLC, PKC-ε and PKC-δ in the IL-1β increase due to LPS/ATP activation and the pathways modulated by PEMFs to reduce this effect were investigated. N9 microglial 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 LPS for 5 hr, in the absence and in the presence of PEMFs. The effect of LPS/ATP on IL-1β production was reduced by inhibitors of AC, PLC, PKC-ε and PKC-δ, suggesting that these signaling pathways are involved in LPS/ATP-induced IL-1β production. None of these inhibitors was able to revert PEMF-induced protective effect (Fig. 17). Furthermore, to examine whether ERK1/2, p38, JNK1/2 MAPK kinases, Akt and caspase 1 pathways were involved in the stimulatory effect mediated by LPS/ATP on IL-1β secretion, N9 were pretreated for 30 min with 1 μM U0126, SB202190, SP600125, SH5 and 10 μM casp 1, inhibitors of ERK1/2, p38, JNK1/2 MAPK kinases, Akt and caspase 1, respectively, before incubation with LPS for 5 hr and ATP for 30 min, in the absence and in the presence of PEMFs. The effect of LPS/ATP on IL1β production was affected by inhibitors of ERK1/2, p38 MAPK kinases, Akt and caspase 1, suggesting that these signaling pathways are involved LPS/ATP-induced IL-1β production. Importantly, SP600125, that did not affect LPS/ATP effect on IL-1β production, was able to revert the protective effect mediated by PEMFs on IL-1β production, suggesting the recruitment of JNK1/2 MAPK kinases in this response (Fig. 18).

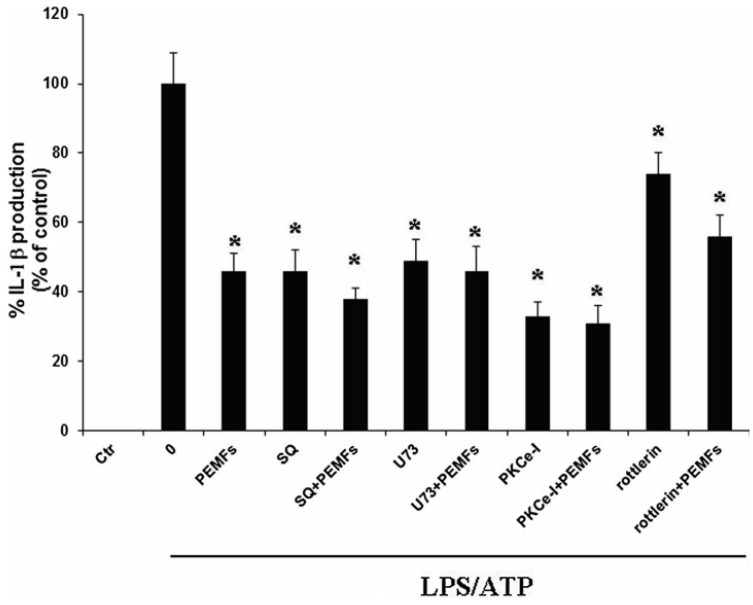

Figure 17. Effect of inhibitors of AC, PLC, PKC-ε, PKC-δ on LPS/ATP-induced IL-1β production from N9 microglial cells, in the absence/presence of PEMFs. N9 cells were incubated with 1 μM SQ22,536, U73122, PKC-ε blocker and rottlerin for 30 min before stimulation with LPS/ATP, in the absence/ presence of PEMFs, for 5 hr/30 min, respectively. IL1β production was evaluated by alphaLISA. *P < 0.01 versus 0 (LPS/ATP alone). Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA followed by Dunnett's test.

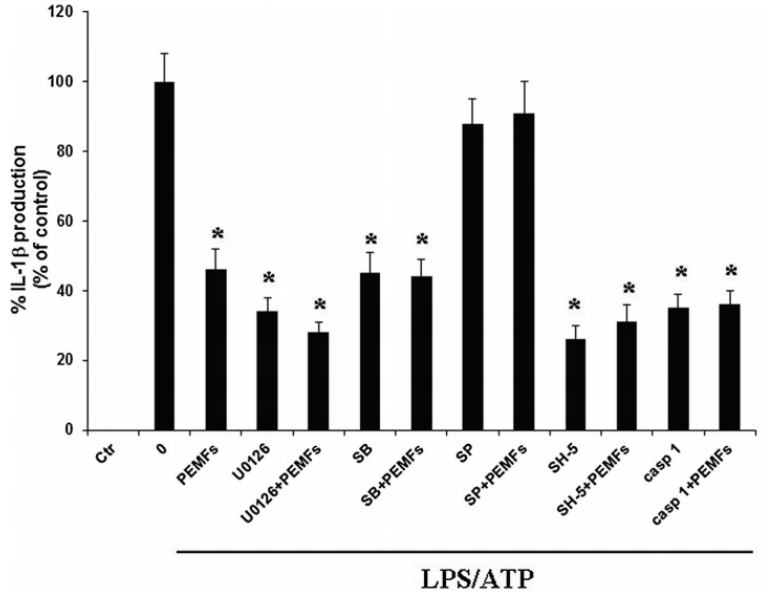

Figure 18. Effect of inhibitors of ERK1/2, p38, JNK1/2, Akt and caspase 1 on LPS/ATP-induced IL-1β production from N9 microglial cells, in the absence/ presence of PEMFs. N9 cells were incubated with 1 μM U0126, SB202190, SP600125, SH5 and 10 μM caspase 1 for 30 min before stimulation with LPS/ ATP, in the absence/presence of PEMFs, for 5 hr/30 min, respectively. IL-1β production was evaluated by alphaLISA. *P < 0.01 versus 0 (LPS/ATP alone). Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA followed by Dunnett's test.

Signaling pathways involved in PEMFs-induced reduction of pro-inflammatory IL-6 in N9 microglial cells

LPS stimulation (1 μg/ml) was able to activate N9 microglial cells mediating a significant increase of the pro-inflammatory cytokine IL-6, after 24 hr of incubation. PEMF exposure was able to reduce the LPS stimulated IL-6 production, thus inducing a protective effect. The involvement of AC, PLC, PKC-ε and PKC-δ in the IL-6 increase due to LPS activation and the molecules modulated by PEMFs to reduce this effect were investigated. N9 microglial cells were pretreated for 30 min with 1 μM SQ22,536, U73122, PKC-ε translocation inhibitor peptide and rottlerin before incubation with LPS for 24 hr, in the absence and in the presence of PEMFs. The effect of LPS on IL-6 production was only slightly affected by inhibitor of PKC-ε but not by blockers of AC, PLC and PKC-δ, suggesting that these signaling pathways are not involved in LPS-induced IL-6 production. Furthermore, none of these inhibitors was able to revert PEMF-induced protective effect (Fig. 19). To examine whether p38, ERK1/2, JNK1/2 MAPK kinases, Akt and caspase 1 pathways were involved in the stimulatory effect mediated by LPS on IL-6 secretion, N9 were pretreated for 30 min with 1 μM U0126, SB202190, SP600125, SH5 and 10 μM caspase 1 before incubation with LPS for 24 hr, in the absence and in the presence of PEMFs. The effect of LPS on IL-6 production was not affected by inhibitors of ERK1/2, JNK1/ 2 MAPK kinases and Akt, suggesting that these signaling pathways are not involved in LPS-induced IL-6 production whilst SB202190 and caspase 1 inhibitor reduced LPS-stimulated IL-6 secretion. However, none of these inhibitors was able to revert PEMF-induced protective effect (Fig. 20). Finally, the effect of CLI-095 was evaluated. This blocker completely reverted (95% inhibition) the LPS-induced IL-6 stimulation, suggesting the involvement of TLR4 receptor in the LPS effect, but PEMF ability to reduce the IL-6 production (34% inhibition without CLI-095) was not dependent by their interaction with it (30% inhibition).

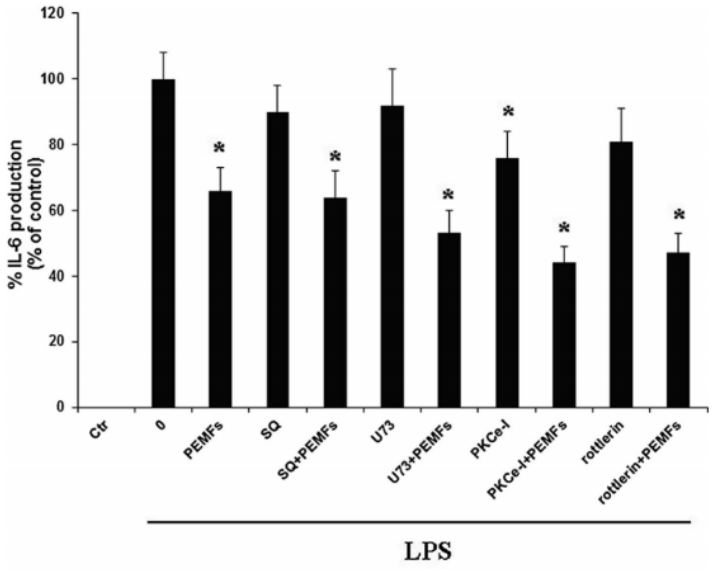

Figure 19. Effect of inhibitors of AC, PLC, PKC-ε, PKC-δ on LPS-induced IL-6 production from N9 microglial cells, in the absence/presence of PEMFs. N9 cells were incubated with 1 μM SQ22,536, U73122, PKC- € blocker and rottlerin for 30 min before stimulation with LPS, in the absence/presence of PEMFs, for 24 hr. IL-6 production was evaluated by ELISA. *P < 0.01 versus 0 (LPS alone). Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA followed by Dunnett's test.

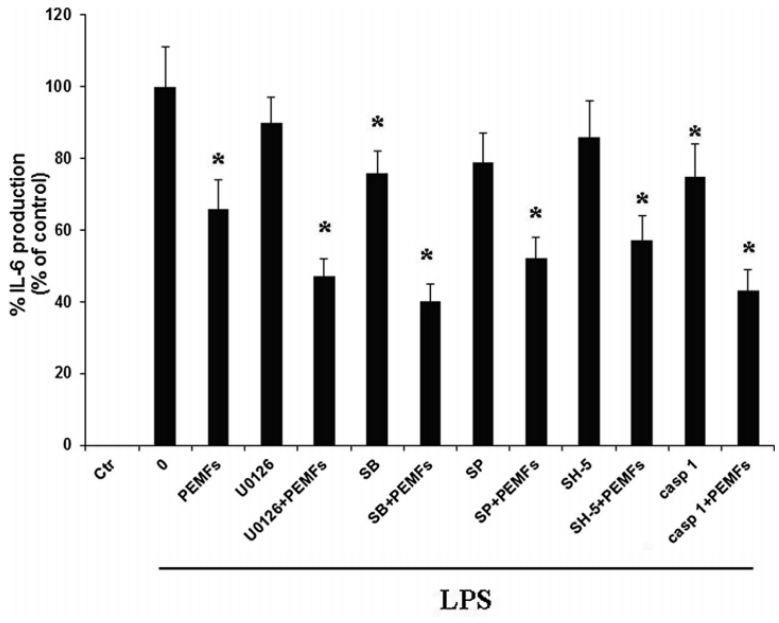

Figure 20. Effect of inhibitors of ERK1/2, p38, JNK1/2, Akt and caspase 1 on LPS-induced IL-6 production from N9 microglial cells, in the absence/presence of PEMFs. N9 cells were incubated with 1 μM U0126, SB202190, SP600125, SH5 and 10 μM caspase 1 for 30 min before stimulation with LPS, in the absence/presence of PEMFs, for 24 hr. IL-6 production was evaluated by ELISA. *P < 0.01 versus 0 (LPS alone). Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA followed by Dunnett's test.

Effect of PEMFs on JNK MAPK kinase

To confirm the involvement of JNK1/2 MAPK kinases in the PEMFs protective effect on TNF-α and IL-1β production induced by LPS, a kinetic study on JNK1/2 phosphorylation was performed. N9 microglial cells were treated for different times (10, 20, 30, 60, 120 min) with LPS, in the absence and in the presence of PEMFs. As shown in Figure 21A, PEMFs in the presence of LPS significantly increased JNK1/2 phosphorylation starting from 20 min with a maximum effect at 30 min, thus confirming the involvement of this kinase in the protective signaling pathway activated by PEMFs. Furthermore, the specificity of JNK inhibitor SP600125 was demonstrated. In N9 cells treated with LPS for 30 min, it strongly reduced LPS-stimulated pJNK as well as it decreased its basal level (Fig. 21B).

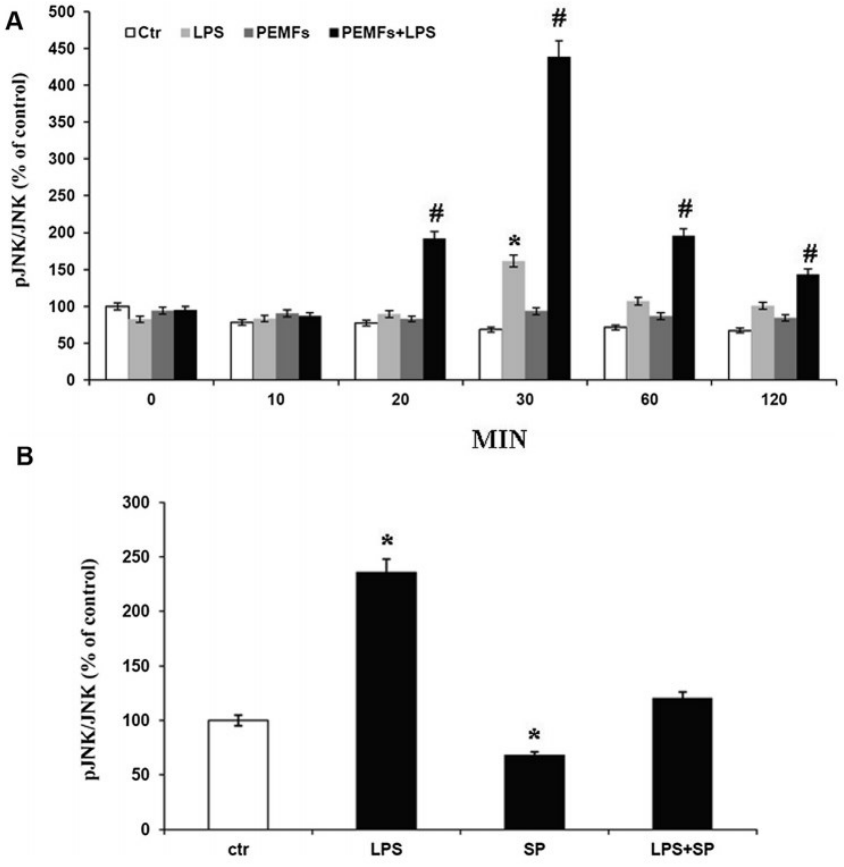

Figure 21. (A) Kinetic of JNK1/2 MAP kinase activation in N9 microglial cells stimulated with LPS, in the absence/presence of PEMFs. N9 cells were incubated with LPS for 0, 10, 20, 30, 60 and 120 min and pJNK1/2 was evaluated by AlphaScreen SureFire assay. (B) Specificity of SP600125 inhibitor on pJNK substrate. N9 cells were incubated with 1 μM SP600125 inhibitor for 30 min in the absence/presence of LPS and pJNK/ JNK ratio was evaluated by AlphaScreen SureFire assay. *P < 0.01 versus Ctr (30 min); #P < 0.01 versus LPS at each time point. Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA followed by Dunnett's test.

Viability of microglial cells following pharmacological treatments

To characterize the vitality of N9 cells following LPS and PEMFs treatments the MTS assay was performed. In particular, microglia were treated with LPS for 24 hr in presence/absence of PEMFs. PEMF treatment by itself did not significantly affect cell viability of microglia (data not shown).

Effect of PEMFs on ROS production

An increase in ROS production was observed following incubation of N9 microglial cells with LPS for 48 and 72 hr. Interestingly, treatment of cells with PEMFs significantly reduced both basal and LPS-stimulated ROS levels. The effect of LPS on ROS production was stronger at 48 hr than at 72 hr, whilst the effect of PEMFs on basal ROS levels was higher following 72 hr (Fig. 22). At 24 h of incubation we did not observe any stimulation of ROS production by LPS, neither any effect of PEMFs on basal ROS levels (data not shown).

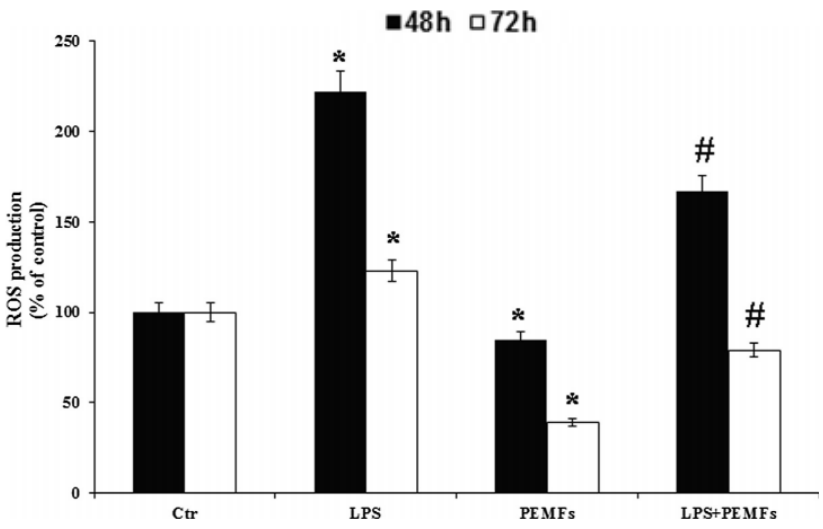

Figure 22. Effect of PEMFs and LPS on ROS production in N9 cells. Microglial cells were incubated in the absence/presence of PEMFs and treated with LPS for 48 and 72 hr. *P < 0.01 versus Ctr (untreated cells); #P < 0.01 versus LPS at each time point. Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA followed by Dunnett's test.

Effect of PEMFs on cell invasion and phagocytosis

In order to evaluate whether PEMFs, in addition to affect cytokine and ROS production in activated N9 microglial cells, could influence also important microglial cell functions aimed to avoid dangerous molecules we addressed our attention to the examination of PEMFs ability to affect cell invasion and phagocytosis. Interestingly, in our experimental conditions, we observed a huge stimulatory effect by LPS on microglia cell invasion, while only a slight increase in cell phagocytosis. Both these effects were abrogated following PEMFs treatment, suggesting an important role of PEMFs in the modulation of inflammation (Fig. 23).

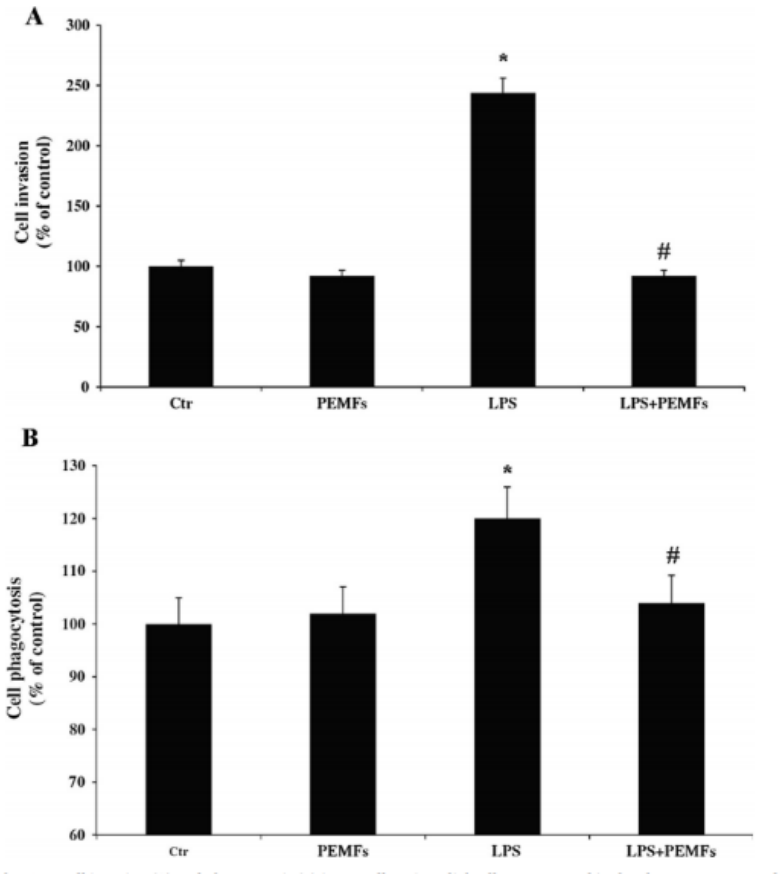

Figure 23. Effect of PEMFs and LPS on cell invasion (A) and phagocytosis (B) in N9 cells. Microglial cells were treated in the absence/presence of PEMFs with LPS for 6 hr (A) or 4 hr (B). *P < 0.01 versus Ctr (untreated cells); #P < 0.01 versus LPS. Means ± S.E. values from four experiments are shown. Analysis was by one way ANOVA followed by Dunnett's test.

DISCUSSION

Stroke is a leading cause of mortality and disability worldwide, with a high incidence and a dramatic public health burden. In particular, ischemic stroke represents the majority of the cases, and often leaves the patients with no independence (Allen and Bayraktutan, 2009).

In this condition, a fundamental role is played by neuroinflammation, that triggers the immune system cells, worsening the lesion (Lehmann et al., 2014; Arnao et al., 2016; Bustamante et al., 2016).

An increasing interest in finding molecules or drugs able to modulate the microglial response, as a potential therapeutic target in diseases characterised by neuroinflammation, such as the acute ischemic stroke in humans, has been growing (Weinstein, 2010).

In fact, the role of activated microglial cells in damaging the brain ischemic area, both affecting the BBB and neuronal cells is well reported. Their activation starts soon after the ischemic event, triggered by both hypoxia and the mediators released from dying neurons. These cells are also involved in the reperfusion-injury damage. In detail, the irroration of the previously ischemic affected area not only restores the oxygen and nutrients, but carries pro-inflammatory mediators and cells, exacerbating the inflammation and so the damages in the area. This problem is serious and is one of the reasons that causes the failure of the artery recanalization, that to date, with the antithrombotic drugs, is the only available therapy (Lai and Todd, 2006).

However, targeting only the pro-inflammatory activities of microglial cells without affecting their neuroprotective action is complicated, and, to date, there are still no available therapeutic molecules or drugs able to do that (Weinstein, 2010).

In this context, PEMFs treatment emerges as safe and non-invasive option, that has been reported to protect neurons and to improve functional recovery after damage. This FDA-approved treatment has shown to exert beneficial effects on several inflammatory disorders including pseudoartrosis, complications related to diabetes mellitus, impaired wound healing, pain, neurodegenerative pathologies and joint diseases (Roland et al., 2000; Canedo-Dorantes et al., 2002; Heden and Pilla, 2008; Weintraub et al., 2009;

Arendash et al., 2010; Cebrian et al., 2010; Goudarzi et al., 2010; Jing et al., 2011; Pan et al., 2013; Varani et al., 2012, 2017; Iwasa and Reddi, 2018).

Promising effects have been reported in bone and cartilage diseases. In particular, PEMFs treatment was able to reduce cartilage degeneration through the inhibition of pro-inflammatory cytokines. Among all a diminishing of IL-1β, IL-6, IL-8, PGE2 in human synoviocytes, chondrocytes and osteoblasts and an increase in IL-10, an anti-inflammatory mediator, was reported (Vincenzi et al., 2012; Yan et al., 2013; Geiseler and Morland, 2018).

PEMFs treatment has been demonstrated to reduce ROS production in human renal proximal tubular cells (Lim et al., 2015) and in osteoblasts, enhancing antioxidative stress response (Ehnert et al., 2017). ROS and inflammation reduction were reported in the damaged spinal cord, too (Wang et al., 2019).

In the context of Parkinson's and Alzheimer's diseases, PEMFs treatment has shown beneficial effects too, improving fine motor function and decreasing fatigue, muscle cramps and tingling, muscle stiffness (Jensen et al., 2018; Malling et al., 2018), and leading to cognitive benefits (Arendash et al., 2010), respectively.

Following arthroscopic surgery, PEMFs treatment ameliorated patient's recovery, reducing the concomitant use of anti-inflammatory drugs, and providing a positive long-term effect (Zorzi et al., 2007; Benazzo et al., 2008). Clinical studies on both bone marrow-derived cells implanted in lesions of the talus and chondrocyte implanted in those of the knee, reported that PEMFs aided patient's recovery and pain control (Cadossi et al., 2014; Collarile et al., 2018). Pain reduction has been observed also in cases of fibromyalgia (Thomas et al., 2007) and rheumatism (Funk, 2018).

As far as the nervous system is concerned, PEMFs have been found to reduce the release of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in LPS-activated N9 microglial cells, suggesting that PEMFs could represent a potential therapeutic approach in cerebral ischemic conditions (Vincenzi et al., 2017).

Finally, PEMFs treatment showed positive effects in cerebral ischemia, leading to a decreased neuronal death in preclinical cellular models as well as in humans (Capone et al., 2009).

Because of these reasons, the aim of this study was to investigate the effects of PEMFs on cytokines secretion and on other specific microglial functions, such as ROS production, migration and phagocytosis. To induce the inflammation-injury, N9 microglial cells were treated with LPS, that has been reported to be a model for neuro-inflammatory studies (He et al., 2017; Ding et al., 2019).

In detail, the pathway involved in the cytokine secretion was investigated, analysing the involvement of AC, PLC, PKC-ε, PKC-δ, p38, ERK1/2, JNK1/2 MAPK, Akt and caspase 1 molecules in both the secretion LPS induced and the protective effect PEMFs mediated on LPS activated cells.

As for the mechanism involved in PEMFs effects, it has been suggested that following a low-energy interaction, the activation of signaling processes may occur and it has been reported that PEMFs may affect membranal protein distribution and phosphorylation (Bersani et al., 1997; Hu et al., 2001). However, the fast and relevant amplification of intracellular signaling makes it easier to identify their activation in place of the initial interaction on cellular membrane (Kapri-Pardes et al., 2017).

The results obtained demonstrated that PEMFs did not alter the microglial cells viability, confirming its safety that has already been reported in neuronal cells. In addition, similarly to the results obtained by Vincenzi and colleagues (Vincenzi et al., 2017), they were able to significantly decrease the production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in activated N9 microglial cells.

It was further investigated the pathway involved. In particular, the LPS-induced TNF-α production was not dependent on AC, PLC, PKC-ε, PKC-δ and Akt activation, but was shown to be mediated by ERK1/2, p38 and caspase 1. These mediators are not involved in the protective effect PEMFs mediated, that is, instead, determined by the activation of JNK1/2 MAPK. In fact, only SP600125 was able to significantly revert the TNF-α decreased induced by PEMFs.

Inhibitors of AC, PLC, PKC-ε, PKC-δ, ERK1/2, p38 MAPK, Akt and caspase 1 reduced IL-1β production, suggesting that LPS/ATP increased IL-1β by activating these enzymes. As seen for TNF- α, JNK1/2 MAPK only is involved in the reduction of this cytokine secretion by PEMFs.

To confirm the involvement of the MAPK mediator, JNK1/2 activation was investigated. Data showed that PEMFs triggered JNK1/2 MAPK, increasing its phosphorylation with a peak after 30 minutes.

Accordingly, stimulation of JNK by PEMFs has been already reported in the peripheral lymphocytes of patients who recently undergo appendectomy, where a general increase in all MAPKs is suggested to be responsible for PEMFs mediated wound healing, fractures and pain modulation effects (El-Makakey et al., 2017). However, in most cases an opposite effect of JNK1/2 increase, that led to an augmented level of pro-inflammatory cytokines (Resnick et al., 2004; Yamasaki et al., 2012) was observed, suggesting the need of further investigations in the beneficial effects of this mediator.

The increase in both TNF-α and IL-1β LPS-mediated was due to the activation of ERK1/2 and p38 MAPKs. These results are in line with literature studies reporting that MAPK phosphorylation, in particular ERK1/2 and p38, plays an important role in LPS-induced microglial signal transduction pathways, that leads to cytokine synthesis (Baldassare et al., 1999; Youn et al., 2013; Kim et al., 2019; Park et al., 2019; Zhou et al., 2019).

The third cytokine investigated was IL-6. In this case too, its increase LPS-stimulated was significantly reverted by PEMFs. In particular, the pathway activated by LSP involved PKC-ε, p38 and caspase 1, while none of the investigated mediators resulted to be involved in the decrease PEMFs mediated. In fact, differently from TNF-α and IL-1β, IL-6 decrease was not affected by SP600125, suggesting a non-involvement of JNK1/2 MAPK.

In addition to cytokines production, activated microglial cells stimulate the pro-inflammatory process through the secretion of ROS and the increase of migration and phagocytosis. These functions are relevant from a clinical point of view considering that they are involved also in the reperfusion-injury damage after a stroke. Specifically, both the hypoxia and necrotic cells, through the release of nucleic acids acting as danger associated molecular pattern molecules (DAMPs), trigger microglial cells, starting the pro-inflammatory response. This process is associated with the recruitment of peripheral immune cells to the ischemic area and with BBB distruption, that consequently leads to the production of other inflammatory mediators, and results in brain edema, hemorrhage and cell death (Kawabori et al., 2015).

Interestingly, PEMFs treatment was able to significantly reduce ROS production. In addition, it also decreased cell invasion and phagocitosys, supporting its anti-inflammatory effects. It is known, in fact, that "phagoptosis", the process that consist in the selective attack and removal of damaged but viable neurons by microglia (Brown and Neher 2012), contributes to brain pathologies with a neuroinflammatory component and to increase the severity of the pathologies themselves.

These effects, at our knowledge, have not been reported in literature following the exposure to a low intensity and pulsed electromagnetic field yet.

In conclusion, this study adds some information about the signaling mechanism involved in the potent anti-inflammatory effects played by PEMFs on TNF-α and IL-1β production in microglial cells as well as new piece of knowledge on their capability to modulate IL-6, ROS production, cell invasion and phagocytosis induced by an inflammatory insult (Fig. 24). Further studies in human models will be necessary to consolidate the potential application of anti-neuroinflammatory functions exerted by PEMFs.

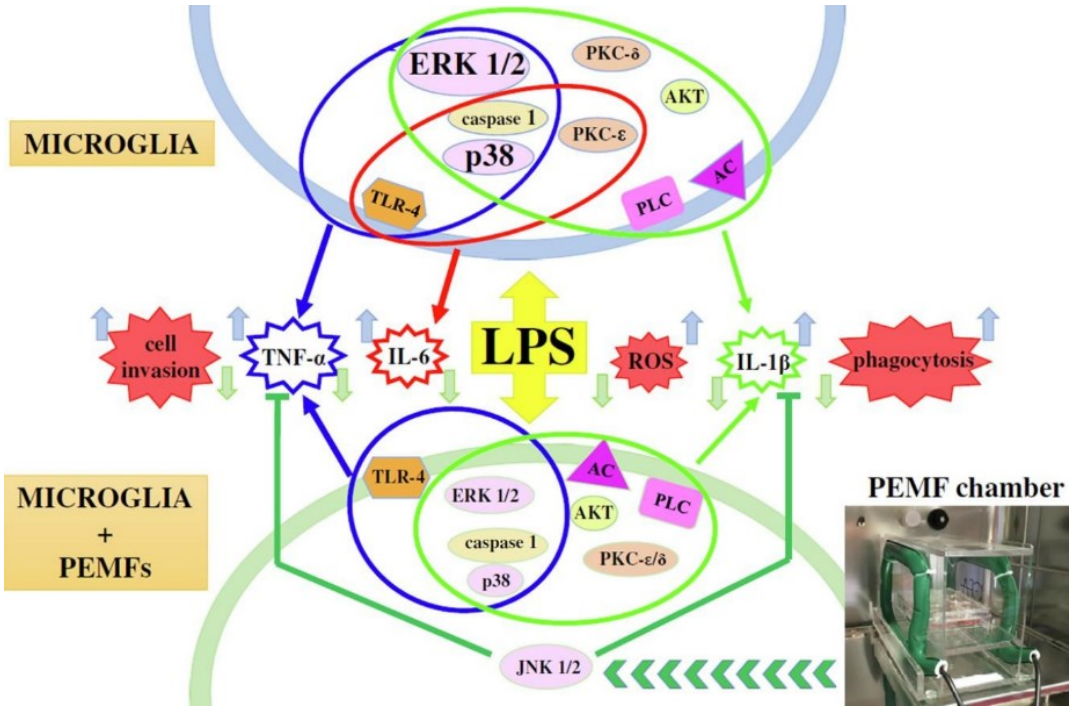

Figure 24. Effects of LPS in treated N9 microglial cells without and with PEMFs. Blue cell: In microglial cells, LPS increases the production of TNF-α (through ERK 1/2, caspase 1, p38, TLR-4), IL-6 (through caspase 1, p38, PKC-ε and TLR-4), IL-1β (through ERK 1/2, caspase 1, p38, PKC- ε and -δ, AKT, AC, PLC), and of some specific cell functions of microglia like ROS generation, cell invasion and phagocytosis. Green cell: Significant effect of PEMFs in the reduction of LPS-stimulated cell-invasion, phagocytosys and ROS, and in the decreased production of the cytokine TNF-α and IL-1β (trough JNK 1/2) and IL-6.

FINAL CONCLUSIONS

Stroke is one of the leading causes of death worldwide and leaves the survivors with permanent disability in 80% of cases.

To date, the only available therapies are the antithrombotic drugs and the artery recanalization. Their effectiveness is still time-limited at the first period after the ischemic event and could lead to no results or further damages (Jauch et al., 2013).

In this context, PEMFs treatment has been reported as a possible option, acting on the different fronts that are responsible of the ischemic damage.

In particular, its protective and anti-apoptotic ability in different cell lines, including neurons in ischemia, and its inflammatory modulating activity, with a decrease in neuroinflammation and an improvement in numerous neurodegenerative diseases has been reported (Grant et al., 1994; Pena-Philippides et al., 2014; Capone et al., 2017; Urnukhsaikhan et al., 2017; Wang et al., 2019).

This work supports the neuroprotective finding described in neurons, demonstrating a decrease in NGF differentiated PC12 cell-death under hypoxic condition after PEMFs treatment. For the first time, the intracellular pathway stimulated by PEMFs was also described: through the activation of phosphoP38, it triggers the chaperone molecule HSP70, the transcriptional factor pCREB and the neurotrophic BDNF.

Interestingly, p38 MAPK is not involved in hypoxic-mediated cell death, but it has been found necessary in the PEMFs mediated protective effect. This result is in line with the one of Sun and colleagues, that, in rats, described firstly an involvement of p38 MAPK in the induction of tolerance by limb ischemic preconditioning to lethal brain ischemic insult (Sun et al., 2006), and later, that this is able to protect the pyramidal neurons in the CA1 region of the hippocampus, reporting a positive involvement of p38 MAPK against the neuronal death that is induced by ischemia (Sun et al., 2010).

Different studies have shown that p38 MAPK modulates HSP70 levels (Uehara et al., 1999; Rafiee et al., 2006; Ko et al., 2008). This mediator is involved in the induction of ischemic tolerance: in particular, its increase was found positive in different studies (Nakata et al., 1993; Yagita et al., 2001). Its beneficial role, due to its augmented level, is

already known in cell survival and recovery after ischemic injury (Aoki et al., 1993a, 1993b; Yagita et al., 2001). Based on these data, it can be hypothesized that PEMFs activate the same pathway in neurons.

This study reported an increase in CREB activation, that is in line with the results obtained by Urnukhsaikhan and colleagues in human BM-MSCs (Urnukhsaikhan et al., 2016). Further, CREB increase was able to modulate BDNF, a well-known mediator for its role in neuronal cell survival and differentiation, formation of the synapses and their plasticity and activity-dependent changes in synaptic structure and function (Park and Poo, 2013). In addition, BDNF inhibits the expression of MHCII and its co-stimulatory molecules B7 and CD40 in microglia cells, keeping their inflammatory pathways under control (Neumann et al., 1998; Wei and Jonakait, 1999).

Interestingly, PEMFs treatment was able to modulate the Bcl-2 family of proteins: in particular, to upregulate the Bcl-2 level, the most known anti-apoptotic molecule, and to downregulate BAD, a pro-apoptotic one. Similar to our findings, in rats' intervertebral discs, PEMFs treatment was found to attenuate the degenerative process regulating the rate of Bcl-2/Bax proteins (Reihani Kermani et al., 2014).

A diminished death could also be obtained through a decreased inflammation, switching the mediators toward an anti-inflammatory direction. In fact, the deleterious role of neuroinflammation in the ischemic area is well reported. One of the most problematic effects that occurs as a consequence of the reperfusion and of the therapeutic artery recanalization is the ischemic reperfusion injury, that consists in the transportation of pro-inflammatory mediators to the damaged area, worsening the situation (Moskowitz et al., 2010).

PEMFs treatment has been reported to decrease the neuroinflammation; therefore, in the second chapter of this thesis the following aspects were investigated: the modulation of cytokine production, ROS generation, cell invasion and phagocytosis by LPS-stimulated microglial cells.

Our results showed that PEMFs treatment is not deleterious for microglial cells, not affecting their viability, supporting the in vivo results of Capone and colleagues that considered PEMFs treatment safe for human patients (Capone et al., 2009).

Further, its ability to reduce the pro-inflammatory cytokines TNF-α, IL-1β, both through JNK MAPK activation, and IL-6 in microglial cells, was showed. These data are in line

with the results obtained in human synoviocytes, chondrocytes, and osteoblasts (Varani et al., 2008; Ongaro et al., 2012; Vincenzi et al., 2013) and demonstrated for the first time JNK involvement in microglial cells.
This could explain the reasons behind the protective effects PEMFs-mediated, as it is well known that a decreased level of pro-inflammatory cytokines and an augmented level of the anti-inflammatory ones is associated to lower infarct size and a better clinical outcome (Pena-Philippides et al., 2014).

Finally, PEMFs treatment was also able to significantly decrease ROS production, as seen in human renal proximal tubular cells (Lim et al., 2015), migration and phagocytosis, suggesting that PEMFs could shift microglial cells toward an anti-inflammatory status.

In conclusion, the data obtained from this study provide evidence that PEMFs treatment could protect the neuronal environment during an ischemic event, both acting directly on neurons and regulating the surrounding microglial cells. Further experiments are needed to fully understand the mechanisms mediated by PEMFs, in order to corroborate their use as an innovative non-invasive alternative strategy after cerebral ischemia.

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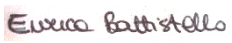

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