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Hypothermia as an Alternative for the Management of Cerebral Ischemia

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1. Introduction

Cerebral ischemia results from the decrease in oxygen and glucose supply by the transient or permanent reduction of cerebral blood flow, triggering excitotoxic, oxidative, inflammatory and apoptotic events which end up in brain tissue death. Cerebral ischemia is one of the leading causes of death in industrialized countries, a medical emergency with few specific treatments available to minimize the acute injury and provide neuroprotection and brain repair. In fact, current therapies are limited to clot removal, aspirin, and decompressive hemicraniectomy for ischemic stroke. To date, alteplase, recombinant tissue-type plasminogen activator (rt-PA) is the only approved therapy for acute ischemic stroke. A relevant concern in stroke research is that despite the increase in pharmacological studies, these treatments have shown to be ineffective or to cause adverse effects. Among more than 700 drugs which have been studied and found to be effective in animal stroke models, yet none has been proved efficacious in clinical studies.

The reduction of cerebral blood flow as a consequence of a thrombus or embolus occlusion results in brain injury with metabolic and functional deficits. The extent of damage depends on the severity and duration of cerebral blood flow decrease; and according to the remaining blood supply, an ischemic core and a penumbra area can be identified.

The core is defined by almost complete energetic failure that ends up in necrotic cell death. Cells in the hypoperfused penumbra are non-functional, however, structural integrity and viability are retained. Experimental and medical evidence indicates that if the blood flow is not restored throughout reperfusion within hours, the penumbral region becomes part of the core. Hence, penumbra is the target to rescue since brain tissue at this region remains potentially viable for 16 to 48 hours, enabling clinicians to intervene and reduce post-stroke disability.

In addition to medication, the development of novel and rational strategies directed to reduce impairments after stroke have been improved. Ischemic preconditioning, electroacupuncture, hypothermia and stem cell therapy are the most relevant non-

pharmacological strategies for the management of the patient who suffers an ischemic stroke, which is at present, one of the most frequent diseases at adult age and the principal cause of disability in many countries.

Hypothermia is considered as one of the most effective options to treat stroke patients in the management of the adverse events taking place in the brain. Hypothermia alters different events in cerebral injury including reduction in metabolic and enzymatic activity, release and re-uptake of glutamate, inflammation, production of reactive oxygen species, blood-brain barrier breakdown and shift of cell death and survival pathways. Although stroke models vary in methodology, several laboratories have consistently shown that hypothermia reduces the extent of neurologic damage and improves neurologic function.

The aim of this chapter is to provide a recent review of basic research in hypothermia treatment. Beside the clinical studies that incorporate hypothermia, numerous efforts have been performed in recent years to understand the mechanisms underlying protection by hypothermia. The clinical and basic research concurrence will allow a better understanding of hypothermia mechanisms in the near future, making its incorporation more efficient as a co-adyuvant in stroke treatment.

2. Protective hypothermia

The reduction of body temperature or hypothermia during an adverse event such as cardiac arrest, cardiopulmonary resuscitation (Hassani, 2010), neonatal hypoxia, hepatic encephalopathy (Barba et al., 2008) and ischemic stroke has been applied in humans as well as in animal models. In all cases, hypothermia has shown to preserve cerebral function. Clinical trials have proved that hypothermia is an effective protector of brain injury (Jacobs et al., 2007), and laboratory animal studies provided a considerable amount of evidence supporting hypothermia protection after focal, global, transient and permanent cerebral ischemia models as well as *in vitro* approaches with ischemia or hypoxia treatments (van der Worp et al., 2010; Yenari & Hemmen, 2010).

The amelioration of ischemia/reperfusion-induced oxidative stress, inflammatory and apoptotic responses are the most promising mechanisms to understand the biological action of hypothermia protection. There is relevant evidence that suggests that hypothermic protection occurs mainly by reducing cerebral metabolism, supporting the protective effects of hypothermia in the different steps of the ischemic cascade as we explain below.

2.1 Hypothermia Induction

Mild ($>32^{\circ}\text{C}$) to moderate ($28\text{--}32^{\circ}\text{C}$) systemic hypothermia has been studied widely. It has been reported that mild hypothermia improves neurological function suppressing apoptosis pathogenesis, and moderate hypothermia limits some of the metabolic responses by altering neurotransmitter release, attenuating energy depletion, decreasing radical oxygen species production and reducing neuronal death and apoptosis.

In clinical stroke, hypothermia is an effective neuroprotective strategy when applied for a long period after the ischemic event, since it has been observed that the optimum conditions for hypothermic neuroprotection are mostly affected by the duration and timing of cooling. Several works have been performed to determine the timing, duration and deepness of

experimental hypothermia. In gerbils subjected to a global ischemia model, the immediate induction or the intensification of hypothermia improved survival rate, suppressed post-ischemic hypoperfusion and prevented vasoconstriction. However, the therapeutic hypothermia time window was narrow, suggesting that it should be induced immediately after the onset of ischemia in order to improve survival (Noguchi et al., 2011).

There are different methods for hypothermia induction in stroke patients and in basic research with *in vivo* models of ischemia. In clinical practice, the main methods to perform hypothermia are surface cooling and endovascular cooling. Both methods have advantages and disadvantages. Surface cooling can be induced with inexpensive methods such as air blankets, alcohol bathing and even fans to decrease temperature. Ice packs, neck bands and head caps are more sophisticated and practical techniques. In addition, surface cooling can be performed in awake patients with ischemic stroke. It is non-expensive, non invasive and allows the use of hypothermia in combination with thrombolytics. In fact, the combination of hypothermia with intravenous tissue plasminogen activator in patients treated within 6 h after ischemic stroke has shown hopeful results (Hemmen et al., 2010). However, complete control of body temperature is not possible with surface cooling, shivering and discomfort of the patient may occur (reviewed in Yenari et al., 2008).

Endovascular cooling seems to be a more efficient way to generate and control hypothermia. Its invasive nature leads to time loss and also requires trained personal in endovascular techniques (Polderman & Herold, 2009). A pilot study with patients with acute ischemic stroke included within 3 h after symptom onset suggests that ice cold saline infusion combined with pethidine and buspirone (to prevent shivering), lowered body temperature to 35.4 ± 0.7 °C in a fast manner and without major side effects. The results of this small uncontrolled case series work, suggest that the induction of hypothermia with an infusion represents a fast approach for induction of hypothermia that could ameliorate the damage caused by the delayed induction observed in the majority of clinical cases (Kollmar et al., 2009). The authors suggest that this rapid induction of hypothermia by ice cold saline infusion is an effective and rapid way to induce mild to moderate hypothermia for stroke treatment in an ambulance car.

The procedure to induce hypothermia also has an important effect in its neuroprotective effect in animal models. Recently, Wang et al (2010) reported the use of systemic, head or local vascular ischemia in rats with middle cerebral artery occlusion. Their results showed that the use of vascular cooling is the most effective procedure to reduce infarct volume as well as in the functional outcome rather than the other two methods (Wang et al., 2010). However in animal models many methods to induce hypothermia are used, including the removal of heating blanket with a spontaneously decrease in temperature (Doshi et al., 2009).

2.2 Alternative agents to induce hypothermia

2.2.1 Helium

Inert gases such as xenon and helium have also been used to produce hypothermia. Helium is considered a “cost-efficient” inert gas with no anesthetic properties, in contrast to the availability and cost of xenon. David et al (2009) have shown that rats subjected to transient middle cerebral artery occlusion and hypothermia generated by helium administered after

reperfusion showed an improvement in neuroprotection. Helium produces cortical protection evaluated by infarct size and reduction of behavioral motor deficits at 25 °C hypothermia but not at 33 °C. The post-ischemic helium hypothermia administration is important as a possible clinical application.

3. Hypothermia amelioration of ischemic damage

3.1 The ischemic cascade

The decrease in oxygen and glucose supply by the transient or permanent reduction of cerebral blood flow in cerebral ischemia triggers a series of excitotoxic, oxidative, inflammatory and apoptotic events known as the “ischemic cascade” which ends up in brain tissue death.

Brain cells are dependent almost exclusively on oxygen and glucose supply for energy production through oxidative phosphorylation. The oxygen and glucose reduction causes accumulation of lactate increasing acidosis. ATP depletion triggers a series of pathologic events including the loss of membrane potential, peri-infarct depolarizations, glutamate and aspartate excitotoxicity, the increase in Ca^{2+} concentration, oxidative stress and free radical generation, protein synthesis inhibition, inflammation and apoptosis. The disruption of ion homeostasis originated by the disturbance of $\text{Na}^+/\text{K}^+-\text{ATPase}$ and $\text{Ca}^{2+}/\text{H}-\text{ATPase}$ pumps, and the reversed $\text{Na}^+-\text{Ca}^{2+}$ transporter, triggers an increase in intracellular Na^+ , Cl^- and Ca^{2+} concentrations, as well as extracellular K^+ . Besides this biochemical response, within minutes after the onset of ischemia, there is an increase in gene expression. Cells respond to stress by adjusting the gene expression program in order to deal with the stress condition, to trigger a recovery process or to lead to signaling for additional tissue injury (Dirnagl, 1999; Durukan & Tatlisumak, 2007).

3.1.1 Brain edema and blood brain barrier breakdown

The effects of hypothermia on the disruption of the blood brain barrier have been implicated in many studies. The role of temperature in blood brain barrier function has been studied in cortex, thalamus, hippocampus and hypothalamus of rats subjected to hyperthermia. Astrocytic activation, a larger content of brain water, Na^+ , K^+ and Cl^- as well as structural abnormalities that suggest brain edema were observed, demonstrating that brain temperature is an important factor in regulating blood brain barrier integrity, permeability and brain edema (Kiyatkin & Sharma, 2009). The effect of temperature in blood brain barrier integrity has also been studied using hypoxia and high ambient temperature to follow the permeability to Na^+ and the expression of the endothelial barrier antigen, a protein associated with blood brain barrier. A clear effect in the increase of Na^+ and a reduction in the endothelial barrier antigen were observed, as well as an exacerbation with hyperthermia (Natah et al., 2009).

The dependence of blood brain barrier integrity and brain edema with temperature has important implications, since even the thrombolytic therapy using rTPA is able to cause hemorrhagic damage. In a work by Hamann et al (2004), it has been proposed that hypothermia could be used as a protection to basal lamina, a component along with the interendothelial tight junctions and perivascular astrocytes of the blood brain barrier. Basal

lamina has the main function of preventing extravasation of cellular blood elements, and the loss of its integrity results in hemorrhage. In order to determine whether hypothermia could maintain microvascular integrity in ischemic stroke, the loss of collagen type IV component of the basal lamina, the non-cellular proteolytic system that degrades basal lamina matrix metalloproteinase (MMP)-2 and MMP-9, plasminogen-plasmin system urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) were determined. Rats were subjected to 3 h ischemia and 24 h reperfusion with the suture model and hypothermia between 32-34°C was applied 30 min before reperfusion. Results were compared with a normothermic group. This work shows that infarct size was considerably reduced in hypothermia treated rats; collagen type IV loss from basal lamina of cerebral microvessels was considerable reduced, as well as MMP-2, MMP-9, tPA and uPA activities, showing that hypothermia preserves microvascular integrity and reduces hemorrhage and the activities of MMP-2, MMP-9, uPA, and tPA (Hamman et al., 2004).

Even that hypothermia has been successful in the protection against neuronal death in several models of ischemia, a recent work using C57BL/6J mice subjected to occlusion of bilateral common carotid arteries, demonstrated that hypothermia induced by the removal of heating blanket with a spontaneously decrease in temperature was an effective protection against neuronal death detected by histological damage and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL). However, brain edema was not prevented by hypothermia treatment (Doshi et al., 2009).

3.1.2 Metabolic downregulation

As mentioned before, in cerebral ischemia two main regions of damage can be defined according to the severity and duration of the cerebral blood flow reduction: 1) The *core*, where complete abolishment of blood supply occurs (less than 12 ml/100g/min) and 2) the *penumbra*, in which collateral blood supply from surrounding arteries assures a flow of approximately 30 ml/100g/min. In normal non pathological conditions cerebral blood flow is decreased under hypothermic conditions, however, the effect of hypothermia in cerebral blood flow during ischemia events is not completely clear since some works support that hypothermia reduces or has no effect in cerebral blood flow and other reports show that it is increased during ischemia. Metabolic suppression has been proposed as one of the most relevant mechanisms underlying the hypothermic treatment. Hypothermia (in the range of 22 to 37 °C) reduces the rate of oxygen consumption fall in body temperature by approximately 5% per degree Celsius. It also decreases glucose consumption and lactate levels (Yenari et al., 2008).

The deepness and extent of hypothermia stimulus has been considered of importance to the outcome and success of therapeutic hypothermia, and the developing methods to monitor and control hypothermia treatments is significant. Single voxel proton magnetic resonance spectroscopy (¹H-MRS) is an important tool to detect metabolites and mechanisms that could be changing during hypothermia. Recently, using ¹H-MRS 7 Tesla MRI scanner, Chan et al (2010) determined the levels of metabolites in response to normothermia and hypothermia. Cortex and thalamus changes of metabolites involved in osmolality, brain temperature and energy metabolism were detected with important implications in the understanding of hypothermia protective mechanisms. For example, it was observed that lactate, the substrate of energy responsible for anaerobic metabolism and anaerobic

glycolysis increased 43% in the cortex during hypothermia. This observation has been considered as a change in energy metabolism associated with neuroprotection which results from an increase in glycolysis and depression of tricarboxylic acid cycle.

Myo-inositol, a metabolite involved in diverse cellular processes such as signal transduction, membrane structure, vesicular trafficking and as an osmolyte modulating cell volume, was increased 21 % in cortex during normothermia. They showed that this technique is able to detect metabolic changes in specific regions of the brain in alive animals in a noninvasively manner. In thalamus, taurine was 16% increased during hypothermia suggesting its role as a regulator of temperature and protecting neurons via its agonistic gamma-aminobutyric acid effect. In the same region choline decreased 29%, and authors suggest that this decrease could imply thermoregulation via muscarinic receptors which act against hypothermia. The main contribution of this work is the demonstration that this noninvasive method can detect changes *in vivo*, of significant metabolites involved in neuroprotective hypothermia (Chan et al., 2010).

Using acid-base related parameters as well as the antioxidant-oxidant effects of deep (21-22°C) hypothermia before acute hypoxic insults in rats, it was observed that during hypothermia mild metabolic acidosis appeared in arterial blood. It suggests that hypothermia induced acidosis contributes to a reduction of potential in liver (Alva et al., 2010). The determination of lactate levels showed that blood lactate increased in normothermia, and hypothermia prevents this increase contributing to the prevention of tissue damage.

3.1.3 Glutamate release and peri-infarct depolarizations

One of the critical steps in cerebral ischemia damage is excitotoxicity by glutamate and aspartate release as a result of membrane depolarization. The activation of glutamate receptors and increase in Ca^{2+} , Na^{+} and Cl^{-} levels initiate molecular events that end in cell death by excitotoxicity. The release of glutamate into the extracellular compartment is one of the early and most intense events of the ischemic cascade. The reduction of glutamate release in hypothermia supports the idea of protection through metabolism downregulation.

It has been observed that the increase in glutamate levels is delayed in the ischemic core as a consequence of hypothermia treatment in permanent focal cerebral ischemia (Baker et al., 1995) and the extracellular glutamate concentration is reduced in the penumbra when analyzed by microdialysis after permanent middle cerebral artery occlusion. This last study suggests that the protection observed by hypothermia probably involves a reduction in the pool of diffusible glutamate in the core but has little effect on glutamate release in the penumbra (Winfree et al., 1996). The increase in glutamate is related to the initiation of peri-infarct depolarizations. Results using the N-methyl-D-aspartate receptor antagonist MK 801 and moderate hypothermia (32-34 °C) have shown neuroprotective effects alone and in combination supporting these observations (Alkan et al., 2001). Several studies have shown that hypothermia decreased glutamate efflux by attenuating the initial rise of extracellular K^{+} and preventing Ca^{2+} accumulation (reviewed in Yenari et al., 2008).

As a result of glutamate excitotoxicity, peri-infarct depolarizations contribute to the increase in infarct volume. The use of temporal NADH fluorescence images to obtain temporal and spatial resolutions to follow the propagation of peri-infarct depolarizations was performed

with spontaneously hypertensive rats subjected to permanent focal ischemia by occlusion of the middle cerebral and left common carotid arteries. Hypothermia (30°C) maintained 2 h and applied before ischemia, showed that hypothermia delays the appearance, however does not modify the dynamics of propagation of peri-infarct depolarizations. The authors suggest that peri-infarct depolarizations could have a greater effect on the infarct area in hypothermic rats (Sasaki et al., 2009) and that the inefficacy to suppress peri-infarct depolarizations is the cause of the absence of hypothermia protection in several models of cerebral ischemia.

3.1.4 Oxidative stress

The generation of reactive oxygen species (ROS), reactive nitrogen species and free radicals is increased as a consequence of ischemic damage and particularly by the restoration of blood supply. Xanthine oxidase, cyclooxygenase, NADPH oxidase, the mitochondrial respiratory chain and the inflammatory response are the major sources of free radicals in cerebral ischemia (Margaill et al., 2005). Reactive species play an important role in both necrotic and apoptotic cell death in cerebral ischemia and reperfusion. ROS generate oxidative stress and triggers tissue inflammation, cause damage to the cellular membrane by lipid peroxidation, DNA damage and disruption of cellular processes. Particularly, superoxide radical has an important responsibility of ischemia damage, since a number of ROS are derived from superoxide.

Using an *in vivo* real-time quantitative superoxide analysis system with an electrochemical sensor previously developed, it has been demonstrated the increase in superoxide in the jugular veins of rats during ischemia/reperfusion in a forebrain ischemia model (Aki et al., 2009). The effect of pre-ischemic hypothermia (32°C) in the generation of superoxide or as a post ischemia treatment (immediately after reperfusion) was determined in the same model. Both pre and post ischemic hypothermia successfully decreased the superoxide generated by ischemic rats. Hypothermia also decreased oxidative stress, early inflammation and endothelial injury markers in both treatments (Koda et al., 2010).

It has been observed that hypothermia maintains the glutathione potential of the liver in an *in vivo* acute hypoxia model, it also avoids the increase in malondialdehyde and prevents tissue damage induced by hypoxia (Alva et al., 2010).

The inhibition of superoxide generation using hypothermia was also evaluated in an insulin-induced hypoglycemia model. They showed that intracellular accumulation of zinc promotes the production of ROS through NADPH oxidase activation after hypoglycemia (Shin et al., 2010). This work also provides evidence that hypothermia could affect other mechanisms such as vesicular Zn^{2+} release and translocation, which affects part of the excitotoxic neuronal death. Hypothermia prevents massive Zn^{2+} release which ends in cell death, and hyperthermia aggravates it, showing that Zn^{2+} release is dependent on temperature (Suh et al., 2004, Shin et al., 2010). However, despite all the evidences of ROS reduction and hypothermia protection, the determination of ROS during the first 60 minutes of ischemia in normothermic and hypothermic conditions using a global cerebral ischemia-reperfusion model, showed that the widely hypothermia protection effect observed does not correlate with the oxidative stress induced by ROS, as observed with electron spin resonance system (Kunimatsu et al., 2001). The use of different ischemia models, time and

temperature are probably the explanation to contradictory results with respect to ROS generation.

3.1.5 Inflammation

Hypothermia attenuates inflammation by suppressing activating kinases of nuclear factor-kappa B (NFkappaB). In a global cerebral ischemia model by bilateral carotid artery occlusion, microglial activation was observed and hypothermia decreased this activation as well as nuclear NFkappaB translocation and activation (Webster et al., 2009). NFkappaB is activated in cerebral ischemia, controlling the expression of inflammatory genes. In a study using middle cerebral artery occlusion by 2 h and hypothermia at 33 °C, it was observed that the decrease in temperature decreased NFkappaB translocation and binding activity. Regulatory proteins such as IkappaB kinase were also affected decreasing its activity, suggesting that hypothermia exerts its protective effect by NFkappaB inhibition (Han et al., 2003).

3.2 Hypothermia and apoptosis signaling pathways

3.2.1 Protein kinases activated by hypoxia

In order to understand the protective mechanisms of hypothermia several efforts have been performed along years. It has been proposed that the mitochondria and the phosphatidylinositol 3-kinase (PI3-K)/Akt (protein kinase B) signaling pathway are determinant for neuronal survival controlling proapoptosis and antiapoptosis in ischemic neurons during stroke. Akt activity has been implicated in the endogenous neuroprotection observed by preconditioning (Miyawaki et al., 2008) and as part of the neuroprotective response to cerebral ischemia (Kamada et al., 2007). Several pharmacological efforts have been performed to target PI3-K/Akt pathway, since it is known that PI3-K/Akt downstream phosphorylated Bad and proline-rich Akt substrate survival signaling cascades are upregulated in surviving neurons in the ischemic brain (Chan et al., 2004).

One of the most relevant efforts is the demonstration that PI3/Akt pathways are involved in neuroprotection by hypothermia. After the distal middle cerebral artery occlusion of rats using intra-ischemic hypothermia (30°C), Zhao et al (2005) observed a reduction in infarct size and the improvement of neurological outcome up to two months. Relevant information was obtained from this work besides observed tissue protection and functional response: 1) decrease of Akt activity observed in normothermic animals after stroke was attenuated by hypothermia; 2) hypothermia improved phosphorylation and attenuates dephosphorylation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and phosphoinositide-dependent protein kinase 1 (PDK1); 3) consequent to the observed tissue protection and the involvement of the Akt pathway, the inhibition of PI3K (an upstream activator of Akt) increases infarct size of hypothermic ischemic rats; 4) phosphorylation of forkhead transcription factor (FKHR) was improved by hypothermia attenuating its apoptotic effects, since dephosphorylated FKHR acts as a transcription factor increasing Bcl-2 interacting mediator of cell death (Bim) and Fas ligand; 5) the nuclear translocation of the transcription factor P-β-catenin, observed in stroke normothermic rats was blocked by hypothermia in the penumbra, but not in the ischemic core, suggesting an important role of β-catenin in stroke excitotoxicity (Zhao et al., 2005).

The same group evaluated the effect of hypothermia in the activation of a kinase implicated in neuroprotection *in vitro*, the epsilon protein kinase C (ϵ PKC), demonstrating that ϵ PKC preservation is an important component in the protective effect of hypothermia. Using the permanent distal middle cerebral artery occlusion plus 1 h of transient bilateral common carotid artery occlusion in normothermic (37°C) and hypothermic (30°C) rats, the neuronal full length ϵ PKC expression and localization was evaluated with immunofluorescence by confocal microscopy and western blot. Normothermic ischemic rats showed a decrease of ϵ PKC in the ischemic core at 4 h after common carotid artery release, hypothermia blocked this decrease. Hypothermia also affects cellular distribution. In non ischemic rats, ϵ PKC is in the cytoplasm of neurons. When cellular ϵ PKC distribution was determined, normothermic ischemic rats showed an ϵ PKC decrease in cytosol as well as in membranal fractions of the ischemic core, blocked by hypothermia. In the penumbra, the membranal ϵ PKC was decreased after the ischemic damage in normothermic rats and hypothermia blocked this decrease. Even hypothermia blocks ϵ PKC cleavage, inhibition of caspase-3 assays showed that this caspase is not involved in this process, suggesting the action of other proteases (Shimohata et al., 2007a).

In contrast to ϵ PKC protective effect, delta protein kinase C (δ PKC) is involved in the tissue damage by ischemia. δ PKC activation depends on catalytic cleavage, phosphorylation and translocation to membranes. The inhibition of δ PKC activity using the specific inhibitor δ V1-1, decreased infarct size after transient cerebral ischemia (Brigth et al., 2004). The translocation to the nucleus and mitochondria, the caspase-3 dependent proteolytic cleavage to generate the δ PKC catalytic fragment (which increased in the membrane fraction, mitochondria, and nuclei); and the release of cytochrome c as earlier as 10 min after reperfusion, are processes triggered by common carotid artery occlusion. Hypothermia blocked all these processes; in fact, the subcellular translocation of the activated δ PKC was attenuated in the penumbra but not in the ischemic core (Shimohata et al., 2007b). All these protective effects were corroborated with the use of a specific δ PKC activator, $\psi\delta$ RACK, in the hypothermic rats.

Primary targets of ischemia-reperfusion injury are vascular endothelial cells through the stimulation of calcium overload, ROS generation and the triggering of inflammatory process, which in turn begin apoptotic programs. In order to determine mechanisms involved in hypothermia protection to ischemia and reperfusion damage, human umbilical endothelial cells were used. Using hypothermia (33°C), a clear reduction in cell apoptosis induced by ischemia/reperfusion was observed. Characterization of this process showed that hypothermia reduces ischemia/reperfusion-induced apoptosis as observed by TUNEL, expression of activated caspase-3 and poly-ADP ribose polymerase (PARP). Hypothermia also reversed Fas/caspase 8 activation pathway and attenuated the Bax/Bcl-2 ratio compared with normothermic cells. Since JNK1/2 and p38 MAPK signaling pathways play an important role in oxidative stress-induced apoptosis, the effect of hypothermia in this pathway was studied, showing that hypothermia inhibits both extrinsic- and intrinsic-dependent apoptotic pathways and activation of JNK1/2 activation via MKP-1 induction (Yang et al., 2009).

3.2.2 Apoptotic proteins

The decrease in apoptosis contributes in a significant manner to hypothermia protection. Ischemia and reperfusion have shown to increase the number of active caspase-3

immunoreactive nuclei, and hypothermia clearly reduced this induction (Kunimatsu et al., 2001).

Recently, Li & Wang (2011) used mild hypothermia (33 °C) in rats subjected to middle cerebral artery occlusion and determined neurological impairment and the expression of Second Mitochondrion-derived Activator of Caspases (SMAC) as an index of cellular apoptosis. Mild hypothermia significantly improved the neurological deficit scores while results from protein and transcript expression of SMAC showed a significantly decrease, suggesting that mild hypothermia could be protecting the functions of cells by attenuating apoptotic death (Li & Wang, 2011).

It has been previously shown that the pro-apoptotic protein SMAC/DIABLO expression is increased in cortex and hippocampus in transient cerebral ischemia as well as in ischemia-reperfusion injury (Saito et al., 2003; Scarabelli & Stephanou, 2004; Siegelin et al., 2005). SMAC increases 3 h after cerebral ischemia with a peak at 24 h. Apparently, hypothermia could be down-regulating SMAC production attenuating caspases activation. Cell apoptosis via SMAC involve mitochondrial and death receptor pathways, inducing changes in mitochondrial membrane permeability and subsequently release membrane proteins, such as SMAC, into the cytoplasm. SMAC leads cells toward apoptosis through apoptosis-related protein. The prevention of loss of mitochondrial transmembrane potential, release of apoptotic proteins (cytochrome c and apoptosis inducing factor [AIF]) and the activation of apoptotic proteins such as caspase 3, as well as the attenuation in the elevation of oxidative stress markers have been observed also in *in vitro* cells with hypothermia in a model of iron and ascorbic acid neurotoxicity (Hasegawa et al., 2009).

Rats subjected to global cerebral ischemia with the four-vessel occlusion model with hypothermia (31-32 °C) and hyperthermia (41-42°C) confirmed the protective effects of hypothermia in the decrease of mortality rate (at 72 and 168 h post reperfusion), and in the increase of surviving neurons in hippocampus under hypothermic conditions. Hypothermia clearly reduced p53 and increased bcl-2 proteins reducing neuronal death. In addition hyperthermia had the opposite effect in the expression of both proteins (Zhang et al., 2010).

3.3 Regulation of gene and protein expression

Hypothermia induces changes in inflammatory, apoptotic and metabolic genes as the result of gene expression regulation.

In general, it is considered that hypothermia downregulates gene expression. However, there are reports that show the upregulation of certain genes, particularly those involved in cell survival. The understanding of gene and protein expression as result of hypothermia in cerebral ischemia could lead to the possible therapeutic target genes or pathways regulated as a result of decreasing body temperature.

Recently, an analysis of gene and protein expression using DNA microarrays and proteomics approach in a 2 h middle cerebral artery occlusion model and mild hypothermia (35°C) has shown that it is possible to determine target molecules. The authors proposed that suppression of neuroinflammatory cascades MIP-3 α -CCR could contribute to the neuroprotective effects of hypothermia and also identified Hsp 70 as a neuroprotective factor stimulated by hypothermia (Shintani et al., 2010; Terao et al., 2009.).

3.3.1 Effect of hypothermia in proteins involved in gene expression

The decrease in temperature and/or the decrease in oxygen concentration involve a series of events that modulate transcription and translation. Novel proteins have been discovered in the last decades. Here we show the role of cold inducible proteins and hypoxia inducible factor-1 as proteins that regulate the efficient transcription and translation of proteins in ischemia and ischemia/hypothermia events.

3.3.1.1 Cold-inducible RNA-binding proteins

Cold inducible proteins have the function to ensure the efficient translation of specific mRNAs at temperatures below the physiological standard. Hypothermia induces the synthesis of amino terminal consensus sequence RNA-binding domain proteins (CS-RBD). The “cold-inducible RNA-binding protein” (CIRP) is one of these proteins, and has been involved in protection as the result of hypothermia treatment in *in vitro* studies. CIRP regulates gene expression at translational level. It binds to the 5'-untranslated region (5'-UTR) or 3'-UTR of specific transcripts, affecting translation and transcript stability (Leonart, 2010).

CIRP has been proposed as a therapeutic target in cerebral ischemia by Liu et al (2010). They determined mRNA expression in hippocampus and cortex of rat brains subjected to hypothermia (30°), cerebral ischemia (by four vessel occlusion model of forebrain ischemia) and hypothermia plus cerebral ischemia by real time quantitative PCR analysis. mRNA CIRP expression was followed at 2, 6 and 24 h showing an increase in cortex after cerebral ischemia with a previous hypothermia treatment. In order to clarify the relationship between CIRP and energy metabolism, they determined lactate and piruvate concentrations, showing that CIRP has a neuroprotective effect in hypothermia; however, it is not related to energy metabolism.

The contribution of CIRP to the neuroprotection observed by hypothermia has also been studied using MEMB5 cells, a neural stem cell line from mouse forebrain (Saito et al., 2010). These cells proliferate in the presence of epidermal growth factor (EGF). EGF deprivation at 37 °C results in apoptosis induction, as well as a decrease of the nestin neural stem cell marker and an increase of the astrocyte marker glial fibrillary acidic protein (GFAP). In contrast, MEMB5 cells at moderate hypothermia prevented apoptosis and decreased the observed GFAP expression of normothermic cells. This observation is important because it suggests that hypothermia prevents neural stem cells differentiation, and it has been hypothesized that the preservation of neural stem cells is one of the neuroprotective mechanisms of therapeutic hypothermia, since it could maintain the capability of cells to differentiate and proliferate after an ischemic event. CIRP mRNA and protein was increased in the MEB5 in hypothermic cells, a response according to previous observations showing that ischemia/reperfusion decreases CIRP mRNA (Xue et al., 1999) whereas hypothermia increases it in an *in vivo* cerebral ischemia model (Liu et al., 2010).

The relevance of CIRP expression was confirmed using CIRP iRNA, increasing apoptosis in hypothermic cells without EGF. This result suggests that the induced CIRP plays the role of a survival factor in neural stem cells. The prevention of apoptosis observed with induced CIRP at hypothermia has been suggested to be the result of the activation of extracellular signal-regulated kinase ERK (Artero-Castro et al., 2009; Sakurai et al., 2006; Schmitt et al., 2007).

3.3.1.2 Hypoxia inducible factor

Hypoxia inducible factor is a transcription factor which binds to hypoxic response elements-driven promoters of genes that mediate adaptive reactions to reduction in oxygen availability. Hypoxia inducible factor is regulated by oxygen accessibility and has been considered as a therapeutic target in cerebral ischemia (Aguilera et al., 2009). Recently it has been reported that persisting low temperature affects its stabilization and protein accumulation. Since the normal regulatory degradation processes of HIF are not affected by hypothermia, it has been hypothesized that probably hypothermia elevates intracellular oxygen tension by decreasing oxygen consumption, suppressing in turn HIF-1 alpha subunit induction. These results were obtained with different cell lines (T98G cells from human glioblastoma multiform, HeLa cells (derived from human cervical carcinoma) and Hep3B cells (derived from human hepatoma) as well as mice subjected to hypoxia and hypothermia (18°C in a mouse incubator). The translation of HIF-1 alpha protein showed to be dependent on time exposure to hypothermia. The down-regulation of HIF protein expression observed with hypothermia has relevant implications in ischemia and hypothermia studies, since this transcription factor is a master regulator of the hypoxic response to oxygen decrease (Tanaka et al., 2010).

3.3.2 Gene and protein expression in CA1 neurons as result of hypothermia

Hippocampal CA1 layer is a region that presents a typical apoptosis cell death after ischemic damage. As a matter of fact, accumulating evidence has indicated that the postischemic DNA fragmentation in the hippocampal CA1 area in experimental ischemic models is a key phenomenon for the delayed neuronal death and is considered as apoptosis. Hypothermia has shown to protect CA1 neurons attenuating the down-regulation of GluR2 mRNA in a model of forebrain ischemia using two days of mild hypothermia induced after 1 h cerebral ischemia, suggesting that the observed attenuation and CA1 neurons protection responds to cooling (Colbourne et al., 2003). Another interesting protein is the β -galactosidase-binding lectin Galectin-3, which has been observed expressed in experimental models of stroke (Walther et al., 2000; Yan et al., 2009) and increased in microglial cells in the hippocampal CA1 layer after a transient ischemic insult. Galectin-3 is a protein involved in apoptotic regulation, inflammation and cell differentiation and used as a marker of activated microglia. After 5 min of bilateral common carotid arteries of gerbils, galectin-3 expression was observed in microglial cells in CA1 region. Hypothermia (31°C) prevents galectin-3 expression suggesting that hypothermia protection occurs through the inhibition of microglial activation and probably by preventing neuronal death (Sato et al., 2011). Even when galectin-3 has been considered apoptotic, its role as an inflammatory mediator in neonatal hypoxia ischemia injury through the modulation of the inflammatory response has been reported (Doverhag et al., 2010).

4. Combined therapies

The preservation of tissue and reduction of brain damage observed during hypothermia and the easiness to achieve and maintain low temperatures, constitute an attractive alternative against stroke. However, because of the complexity of the pathophysiological mechanisms involved in the ischemic cascade, it is common to observe the use of one or two drugs

besides hypothermia in the search of a neuroprotective compound capable of blocking the metabolic insult. Combination therapy is based in the extension of the therapeutic window of hypothermia using pharmacotherapy and establishes an interesting approach in hypothermia research.

4.1 Hypothermia, tirilazad and magnesium

The combined administration of the antioxidant tirilazad, magnesium and hypothermia has shown that the hypothermia protection increases with the use of pharmacotherapy, extending the therapeutic window of hypothermia treatment. Zausinger et al (2003) used hypothermia (2 h, 33°C) at 0, 1, 3 and 5 h after transient focal ischemia induction combined with two administrations of tirilazad (3 mg/kg) and magnesium (1 mmol·L⁻¹·kg⁻¹) in 1 h intervals. Infarct volume was reduced by 74%, 49% and 45% when hypothermia was applied at 0, 1 and 3 h. No improvements were observed at 5 h. The same combination therapy was performed in a permanent ischemia with middle cerebral artery occlusion. Two h at 33 °C hypothermia and two times of drug administration (30 min before and 1 h after middle cerebral artery occlusion) showed a 52 % infarct size reduction after 6 h occlusion. Interestingly, a separate group with 7 days of permanent middle cerebral artery occlusion was followed daily with neurological tests and body weight. Even high mortality occurred in this group, neurological recovery was observed in survivor rats, as well as a decrease in infarct size (Schöller et al., 2004).

Moderate hypothermia (30°C) has shown higher protection than mild hypothermia (33°C), however moderate hypothermia is associated with severe side effects. Nonetheless, the combined effect observed with mild hypothermia, magnesium and tirilazad showed a comparable protection with that observed using moderate hypothermia, suggesting that combination therapy could be a promising approach in clinical applications.

4.2 Hypothermia and magnesium

Combination therapy with magnesium has generated controversy, because magnesium treatment alone has shown to be ineffective in normothermic rats subjected to ischemia (Zhu et al., 2004, a and b). The quantification of infarct volumes at magnesium 360 or 720 µmol/kg in rats subjected to middle cerebral artery occlusion showed that the 360 µmol/kg dose reduces the striatal infarct volume by 32 %. The authors observed that mild spontaneous hypothermia was responsible of the observed neuroprotective effect of magnesium (Campbell et al 2008a).

The therapeutic time window of combined magnesium and mild hypothermia treatment was determined after permanent middle cerebral artery occlusion. The administration of a magnesium sulfate infusion (360 µmol/kg, then 120 µmol/kg/h) and mild hypothermia (35°C) after 2, 4 or 6 h ischemia showed that combination therapy considerably reduced infarct volumes at 2 and 4 h but not at 6 h, supporting the use of combined therapy even at delayed hours after ischemia onset (Campbell et al 2008b).

Although the controversy in the use of magnesium and hypothermia in cerebral ischemia exists, a lot of experimental evidence demonstrates its efficacy in laboratory studies and it is necessary to perform more clinical trials to translate this combinatorial therapy to clinical

use. Actually, combined mild hypothermia (35°C) and magnesium is recognized as a neuroprotective treatment able to minimize ischemic damage, even in delayed treatments (Meloni et al., 2009).

4.3 Hypothermia and citicoline

Hypothermia has been combined with other compounds. One of them is citicoline, an endogenous compound that has shown to stabilize membrane function and reduce free radical generation during ischemia (Rao et al., 2000).

Mild hypothermia combined with citicoline resulted in an additive effect in attenuating apoptosis in focal cerebral ischemia/reperfusion injury. Hypothermia (34±1°C) during middle cerebral artery occlusion (2 h) followed by 24 h reperfusion was used in combination with 4000 mg/kg *i.p.* citicoline. Combined therapy with citicoline and hypothermia resulted in reduced apoptotic cell death before the development of the apoptosome, thus preventing apoptosis and neuronal damage. Bcl-2, caspases 3 and 9 as well as Bax proteins were immunohistochemistry tested, showing that the use of citicoline with hypothermia is more effective than citicoline or hypothermia used alone. A decrease in cerebral injury was observed and apparently, the tissue protection is due to the suppression of apoptotic processes (Sahin et al., 2010).

4.4 Hypothermia and ginkgolides

Despite the neuroprotective role of hypothermia, the combined use of hypothermia with protective compounds not always has a synergistic effect. This is the case of hypothermia and ginkgolides in astrocytes subjected to ischemia and reperfusion in which hypothermia attenuates, rather than enhances, the protective effect of ginkgolides on astrocytes from ischemia and reperfusion-induced injury. Co-treatment with different doses of ginkgolides at 32 and 28 °C hypothermia during 24, 48 and 72 h before 24 h ischemia followed by 24 h reperfusion showed that the use of ginkgolides without hypothermia treatment had an improvement in cell viabilities and in anti-apoptotic properties, and this protective effects were not observed in the co-treatment (Fang et al., 2009).

4.5 Hypothermia and xenon

Xenon has been considered a great promise used as a neuroprotectant in *in vivo* and *in vitro* studies. Combined therapy with hypothermia in a neonatal rat hypoxia-ischemia model has shown that the combination of 50% xenon and hypothermia of 32°C has a protective effect in the restoration of long-term functional outcomes and global histopathology, showing that the combined xenon/hypothermia has a greater protection (Hobbs et al., 2008).

5. Conclusion

Despite all the efforts to develop pharmacological treatments to contend with cerebral ischemic damage, nowadays, there are no efficient treatments to deal with this pathology. Non-pharmacological treatments emerge as reasonable approaches to compete against ischemic damage.

Hypothermia has shown positive results in cardiac arrest and neonatal hypoxic ischemia and in patients with acute brain injury. However, there are no large clinical stroke studies that could assist in the comprehension of the hypothermia role in stroke. In laboratory studies, hypothermia is a consistent protective agent. The present chapter shows the contribution of hypothermia research in the last years to understand its participation during the different stages of the ischemic damage cascade. Blood brain barrier integrity, metabolic rate decrease, redox state changes, inflammatory, apoptotic, signalling and gene regulation are targets where hypothermia protection participates. Hypothermia is affected by diverse factors such as timing, duration and deepness. All these factors contribute to disparity in obtained results. However, it is clear that this research is necessary in order to determine the exact targets, the time when hypothermia begins and duration of hypothermia. Additionally, since brain ischemia is a multifactorial problem and hypothermia has demonstrated to decrease damage in several stages of the process, combination therapy is an alternative to improve treatments by the extension of the therapeutic time window of hypothermia protection.

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7. References

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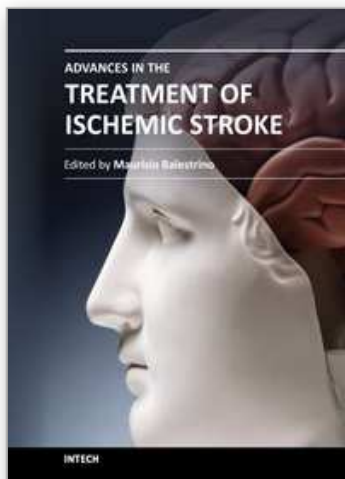
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In recent years research on ischemic stroke has developed powerful therapeutic tools. The novel frontiers of stem cells therapy and of hypothermia have been explored, and novel brain repair mechanisms have been discovered. Limits to intravenous thrombolysis have been advanced and powerful endovascular tools have been put at the clinicians' disposal. Surgical decompression in malignant stroke has significantly improved the prognosis of this often fatal condition. This book includes contributions from scientists active in this innovative research. Stroke physicians, students, nurses and technicians will hopefully use it as a tool of continuing medical education to update their knowledge in this rapidly changing field.

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