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REVIEW ARTICLE

# Neuroprotective Role of Hypothermia in Hypoxic-ischemic Brain Injury: Combined Therapies using Estrogen

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**Abstract:** Hypoxic-ischemic brain injury is a complex network of factors, which is mainly characterized by a decrease in levels of oxygen concentration and blood flow, which lead to an inefficient supply of nutrients to the brain. Hypoxic-ischemic brain injury can be found in perinatal asphyxia and ischemic-stroke, which represent one of the main causes of mortality and morbidity in children and adults worldwide. Therefore, knowledge of underlying mechanisms triggering these insults may help establish neuroprotective treatments. Selective Estrogen Receptor Modulators and Selective Tissue Estrogenic Activity Regulators exert several neuroprotective effects, including a decrease of reactive oxygen species, maintenance of cell viability, mitochondrial survival, among others. However, these strategies represent a traditional approach of targeting a single factor of pathology without satisfactory results. Hence, combined therapies, such as the administration of therapeutic hypothermia with a complementary neuroprotective agent, constitute a promising alternative. In this sense, the present review summarizes the underlying mechanisms of hypoxic-ischemic brain injury and compiles several neuroprotective strategies, including Selective Estrogen Receptor Modulators and Selective Tissue Estrogenic Activity Regulators, which represent putative agents for combined therapies with therapeutic hypothermia.

**Keywords:** Hypoxic-ischemic brain injury, neuroprotective treatments, selective estrogen receptor modulators, selective tissue estrogenic activity regulators, therapeutic hypothermia, combined therapies.



## 1. INTRODUCTION

The Central Nervous System (CNS), due to its own functional characteristics, is susceptible to changes in energy levels, especially in oxygen (O2) and glucose concentration [1]. Oxygen and glucose consumption by the brain is 20 and 25 %, respectively [2, 3]. This high energy demand is attributed to functions such as the recycling of neurotransmitters, ion transport and the synaptic activity carried out by brain cells [2]. Therefore, an efficient metabolism of the brain allows an optimal neuronal functioning. Hypoxic-ischemic (HI) brain injury is mainly characterized by decreasing O2 concentration and blood flow, which lead to an inefficient supply of nutrients to the brain. This pathophysiological conditions lead to cell death by energy depletion and increased free radical generation [4], as it is observed in perinatal asphyxia (PA) and ischemic-stroke (IS) [5-7]. PA and IS represent one of the main causes of mortality and morbidity in children and adults worldwide [8, 9]. Therefore, knowledge of underlying mechanisms triggering these insults becomes essential for the establishment of efficient treatments.

Several neuroprotective strategies have been tested, including Selective Estrogen Receptor Modulators (SERMs) and Selective Tissue Estrogenic Activity Regulators (STEARs), which have shown the same benefits as estrogen, including the decrease of reactive oxygen species (ROS), maintenance of cell viability, mitochondrial survival, among others; without its negative side effects [10-12]. However, these therapies remain inconclusive, representing a traditional approach of targeting a single factor of pathology.

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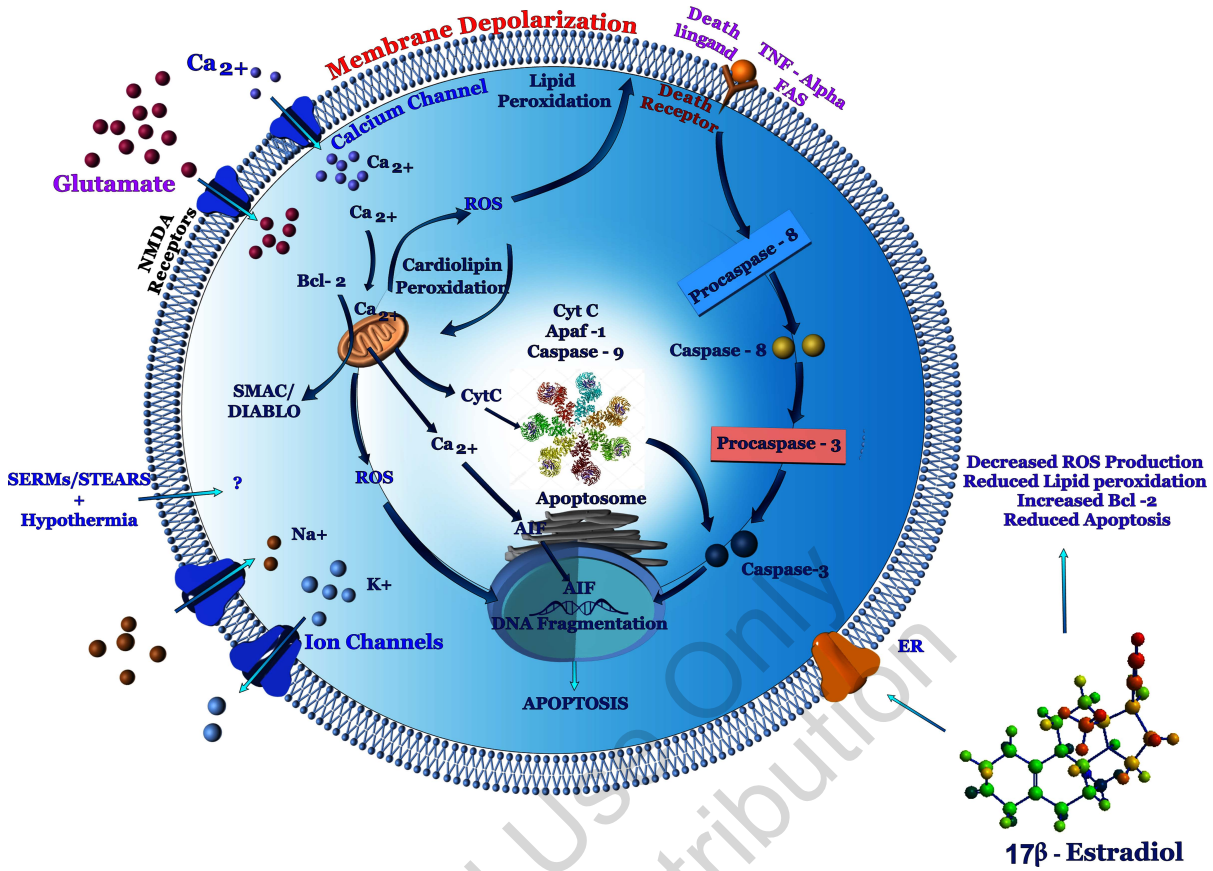


**Fig. (1). Mechanisms of brain damage in hypoxia-ischemia and role of Neuroactive steroids and hypothermia.** Calcium, $Ca^{2+}$; Cytocrome-C, Cyt C; Estrogen receptor, ER; Reactive Oxygen species, ROS; Selective estrogen receptor modulators, SERMs; Selective tissue estrogen activity regulators, STEARs, Sodium, $Na^{+}$; Potassium, $K^{+}$. (*The color version of the figure is available in the electronic copy of the article*).



Currently, the implementation of combined therapies has gained greater relevance [13]. Some combined therapies include therapeutic hypothermia and a complementary neuroprotective agent, since the former, which is the only treatment used in the clinical practice, does not exert complete neuroprotection [14]. In this sense, the present review summarizes the underlying mechanisms of HI brain injury and compiles several neuroprotective strategies, including SERMs and STEARs, which represent promising agents for combined therapies with therapeutic hypothermia.

## 2. MECHANISMS OF BRAIN DAMAGE IN HYPOXIA-ISCHEMIA

HI triggers an initial response immediately after the insult, followed by a secondary response some hours later (Fig. **1**), and a third phase of persistent effects which lasts several years [15]. Global hypoxia affects the cerebral cortex, the thalamus and the basal ganglia, causing damage to deep gray matter [16]. While the complete pathogenic pathways of HI are not fully described, some mechanisms like increased glutamate, calcium overload, mitochondrial dysfunction and oxidative stress have been proposed to contribute to generate neuronal damage [17].

Primary response depends on the energetic failure causing primary brain injury. This is characterized by a reduction of energy supply, generating an accumulation of ROS *via* lactate production augment, and making the cell susceptible to oxidative stress and mitochondrial dysfunction [16]. Low levels of adenosine triphosphate (ATP) derived from this energetical failure affect the integrity of cell membrane. Ion flux of sodium ($Na^{+}$)/potassium ($K^{+}$) is altered by the $Na^{+}/K^{+}$ pump dysfunction. In addition, calcium ($Ca^{2+}$) enters easily to the cell causing membrane depolarization and inhibiting glutamate storage inside the cell, being therefore accumulated in the extracellular space [18]. The second phase of the injury is caused by the recovery of blood flow and the reestablishment of brain metabolism, which are characterized by an inflammatory response, excitotoxicity and oxidative stress. These processes are responsible for cell death after HI and continue exerting deleterious effects for years [16].

### 2.1. Cell Death During the Second Phase of Injury

Apoptosis and necrosis are cell death pathways displayed after HI (Fig. **1**). However, apoptosis is the most common one in the young brain unchained by mitochondrial failure [19]. Apoptosis can follow two pathways: the extrinsic pathway, triggered by external signals like Tumor Necrosis Factor- alpha (TNF-α) and Fatty Acid Synthase (FAS); and the intrinsic pathway, mediated by internal factors such as DNA damage or cell stress [20].

The extrinsic pathway is involved in the action of Caspase 8 and 10, which activate caspase effectors directly, interacting with the intrinsic pathway and triggering a permeabilization of mitochondrial membrane [20, 21]. The intrinsic pathway is mediated by the release of apoptotic factors such as cytochrome-c (cyt-c), second mitochondria-derived activator of caspase/Direct inhibitor of apoptosis-binding protein with low pI (Smac/Diablo), Apoptosis Inducing Factor (AIF), endonuclease G (endoG), Serine protease HTRA2, mitochondrial **(**Omi/HtrA2) after permeabilization of cell membrane [20,21]. These apoptotic factors may trigger cell death processes, which could be mediated by caspase-dependent pathways. Cyt-c interacts with Apoptosis protease-activating factor-1 (Apaf-1), creating the apoptosome. Smac/Diablo interacts with apoptosis inhibitors. AIF and endoG also act through a caspase- dependent pathway. These apoptotic factors are translocated to the nucleus, causing nuclear fragmentation [20, 21]. Hence, permeabilization of mitochondrial membrane has been proposed as a marker of a point of no return in HI.

### 2.2. Excitotoxicity and ROS Production

Another agent responsible for brain damage caused by HI is the increase in extracellular glutamate concentration as mentioned above. Glutamate activates N-Methyl-D-Aspartate (NMDA) receptors, causing the accumulation of $Ca^{2+}$ and nitric oxide (NO), and the production of ROS in turn (Fig. **1**) [22].

One of the main organelles affected by energy failure is mitochondria, which plays a key role in cell death due to the control of energy metabolism, production of ROS, and the release of apoptotic factors into the cytoplasm. The most prominent pro-apoptotic factor is cyt-c [23]. Apoptosis-inducing mitochondrial proteins such as endoG, Smac/Diablo and Omi/HtrA2 have also been described playing an important role in apoptosis regulation [24]. The release of these pro-apoptotic factors is not necessarily mediated by Mitochondrial Permeability Transition (MPT) pores, indicating that changes in mitochondrial membrane potential ($\Delta\psi m$) are directly related to necrosis and apoptosis [25]. At this stage, cell apoptosis is beginning, specifically mitochondrial apoptosis, which is characterized by an excessive release of $Ca^{2+}$ , hyperpolarization of $\Delta\psi m$, and an excessive production of ROS [26].

Another consequence of cell death caused by oxidative stress is cardiolipin peroxidation, one of the most critical targets [27]. Cardiolipin is a unique phospholipid, which is found mostly in the inner mitochondrial membrane, where it has a very close association with the components of oxidative phosphorylation [27]. Cardiolipin plays a crucial role in the function of cytochrome-C, cytochrome- C oxidase and other phosphorylation complexes. This is required, therefore, for an optimal functioning of complex I (NADH: ubiquinone reductase), complex III (NADH: ubiquinone cytochrome-C oxidoreductase), complex IV (cytochrome-C oxidase) and complex V (ATP synthase) [28]. An alteration in the structure of cardiolipin is responsible for mitochondrial dysfunction. Therefore, the release of cytochrome-c depends on the integrity of cardiolipin, which is highly sensitive to peroxidation by ROS, due to its high content of fatty acids [28].

### 2.3. Glial Cells and Neuroinflammation

Glial cells play differential roles in HI brain injury, mainly in inflammation. If glial response becomes chronic, it may potentiate the mechanisms of damage mentioned above, due to the secretion of a large amount of proinflammatory cytokines and ROS.

#### *2.3.1. Microglia*

Microglia, the immune cells of the CNS, are the first to be activated after HI. Microglia migrate to the place of injury, where they change their morphology to amoeboid-like functional cells, acting in conjunction with monocytes and macrophages [29-31]. Microglia present a dual behavior on inflammation caused by HI. Microglial M1 phenotype releases proinflammatory agents to the environment such as ROS, cytokines Interleukin- 1beta, Interleukin-6 (IL-1β, IL-6, TNF-α), glutamate, and nitric oxide, creating a cytotoxic environment which triggers cell death [29-31]. In contraposition, microglial M2 phenotype is responsible for mediating an anti-inflammatory response, exerting neuroprotective properties by the release of anti-inflammatory agents and growth factors (insulin-like growth factor (IGF), transforming growth factor (TGFb), Glial cell-derived neurotrophic factor (GDNF), and Interleukin-10 (IL-10)) [29].

#### *2.3.2. Endothelial Cells*

The extent of injury noted in HI is not only determined by the biochemical cascades that trigger the apoptosis-necrosis continuum of cell death in the brain parenchyma, but also by the pro-inflammatory factors of the Blood Brain Barrier (BBB), such as the endothelial cells [32]. Endothelial cells can sense variation in the Parcial Oxygen Pressure ($PO_2$) through different mechanosensors. Then, they can adapt their metabolism to maintain ATP production, switching into a hypoxic metabolism. In this way, endothelial cells augment the production of ROS by making the respiratory chain slower, reduce the cytochrome-c capacity in order to trap $O_2$, and alter the cellular redox potential [33].

#### *2.3.3. Astrocytes*

In the last 20 years, astrocytes have been granted multiple functions, such as providing support to neurons, helping in the maintenance of the neural microenvironment, and regulating cerebral blood flow, which is necessary for the adequate function of neurons [2, 34]. Astrocytes also contribute to the accurate metabolic function of the brain [35]. These glial cells take glucose from blood vessels and provide energy metabolites to the neuron [36]. In addition, astrocytes provide lactate to neurons as a substrate for the citric acid cycle, supplying in this way their energy requirements [37].

However, the role of astrocytes in injuries such as HI is not fully elucidated [38]. Like microglia, astrocytes respond differently depending on the severity of the injury. Immediately after HI, astrocytes exhibit an activated state, which eventually leads to the formation of a glial scar [39, 40]. Astrocytes may exacerbate cytotoxicity death after the secretion of inflammatory cytokines such as IL-1, IL-6, interferon-γ, and TNF-α; and help the migration of immune cells to the CNS by the secretion of chemokines [30]. On the other hand, astrocytes play a neuroprotective effect by promoting toler-

ance to cerebral ischemic injury [41] and attenuating neuroinflammation [41, 42].

## 3. EXPERIMENTAL MODELS

For the study of HI, *in vivo* and *in vitro* models are used (Table **1**). The murine unilateral common carotid artery occlusion (UCCAO) represents a widely used model of HI. The UCCAO is followed by an exposure to an $O_2$ atmosphere of 8% for 1-3 hours [43], reproducing the anatomical injury caused by HI in neonates, which damages gray matter in hippocampus, thalamus and basal ganglia, as well as in white matter [43,44]. Similarly, it reproduces metabolic damage in parameters such as cerebral acidosis, decreased cerebral blood flow, and decreased glucose uptake [29], and it is useful for studying the neuroprotective effect of hypothermia [45, 46]. Bilateral common carotid artery occlusion (BCCAO) is also used to accentuate damage in white matter [47, 48]. UCCAO and BCCAO have been also used in larger animals such as primates, sheep, pigs and rabbits in order to better replicate the conditions of a human fetus with HI, with the disadvantage of not permitting the performance of behavioral tests [29, 44, 49, 50].

Another murine model of HI excludes ligation of the common carotid, causing damage only by $O_2$ deprivation. This model is used to reproduce milder and severe lesions, in order to investigate the biochemical alterations of the brain [29]. Under this concept there are several methods, in which the times of exposure to hypoxia and the percentage of $O_2$ are variable [29]. Concentrations vary from 2.6% $O_2$ for 30 min [51, 52], and 5% $O_2$ / 95% nitrogen ($N_2$) for 15 min [53]. Other methods provide $O_2$ atmospheres that decrease from 7% to 4% [54], or 9% $O_2$ for 1 hour [55]. There are other models of PA in rats that differ from the previous ones by the way in which the whole procedure is performed. Rat pups inside the uterine horns are subjected to a severe asphyxia by submerging them in a water bath at 37°C for 19 min [56], reproducing in this way a intrauterine global asphyxia, which is highly prevalent in humans. In addition, this model has been widely used for studying synaptic alterations caused by PA [57]. The lack of methodological homogeneity constitutes a disadvantage since it makes difficult to contrast the results in an optimal way [29, 44, 49, 50].

### 3.1. *In Vitro* Approaches

The different methodological limitations of *in vivo* models make *in vitro* models relevant. To replicate the conditions that occur in the presence of deprivation or decrease in glucose and $O_2$ levels such as those present in HI, several studies have proposed a model of $O_2$ and glucose deprivation (OGD) (Table **1**) This model has the ability to adjust to specific research needs and the versatility of including different cell lines, making possible the study of molecular and biochemical mechanisms of HI injury. However, methodological differences have been found in the implementation of this model, especially in the exposure time of hypoxia and reoxygenation, making this model dependent on the specific conditions of the tissue or cells used. Different studies suggest hypoxia times of 1, 1.5, 2.3, 4, 6, 8, 16, 48 h and reoxygenation of 1, 2, 8, 12, 24, 48h [78-81, 84, 85, 87, 106].

Another methodological approach used to study the effects of hypoxia *in vitro* include the use of chemical agents known as hypoxia-mimetic agents (HMAs). These are based on producing at molecular level the effects caused by the low concentration of $O_2$, mainly the effects involved in the expression of Hypoxia-inducible factor-1 (HIF-1) [92, 93]. The activation of this factor depends on $O_2$ concentration, and HIF-1 is involved in several cellular processes that trigger hypoxia [107-112]. Hypoxia can be induced in normoxia by different types of compounds, among them: iron chelators, such as desferrioxamine (DFO) [113, 114] and divalent metal ions such as cobalt and nickel [114]. These agents can inactivate different enzymes associated to hypoxic processes substituting the ferrous group in regulatory dioxygenases. In addition, cobalt and nickel block the delivery of ferrous ions into cells since they bind much more tightly to the membrane transporter DMT-1 (Divalent Metal Transporter 1) in comparison to iron ions [114].

Within the HMAs, multiple compounds have been evaluated among these: 2-aminoanthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, cobalt sulfate, dibenz(a,h)anthracene, 7-diethylamino-4-methylcoumarin, 7,12-dimethylbenz(a) anthracence, iodochlorohydroxyquinoline, o-phenanthroline, prednisone ,salicylazosulfapyridine and triamterene, among others [114]. However, DFO and cobalt chloride ($CoCl_2$) are the most widely used compounds in *in vitro* models. DFO and $CoCl_2$ are also used in different *in vivo* experimental models [94, 95, 97-99-101, 104, 115-117]. DFO has also shown protective properties against hypoxia [98], including *in vitro* models of HI (Hamrick *et al*., 2005). It is administered in preconditioning models in order to attenuate the damage [99, 101, 102]. Similarly, in murine models of PA it has been used as a combined treatment with erythropoietin, reducing the number of cleaved caspase 3 (+) cells [103]. In addition, it has been used as a neuroprotective agent in dopaminergic neuronal death [96].

Summarizing, these compounds have a wide versatility to be used in HI models. However, the use of HMAs to reproduce an HI event presents limitations, one of which is related to the regulatory mechanisms at transcriptional and post-transcriptional levels, since they use distinct regulatory mechanisms through the interplay between DNA and mRNA elements [115]. These limitations should be taken into account at the moment of implementation.

## 4. NEUROPROTECTIVE AGENTS TESTED IN HI

Neuroprotective agents tested in HI are classified according to their mechanism of action: antioxidation, anti-inflammation, anti-excitotoxicity and anti-apoptosis [19]. Allopurinol and aminoguanidine are antioxidant; ammonium pyrrolidinedithiocarbamate (PDCT), anti-inflammatory; memantine and topiramate, antiexcitotoxic; and indomethacin, antiapoptotic [19].

Within most studied therapies, Xenon appears as a noble gas used as an anesthetic agent, showing neuroprotective effects in *in vitro* and *in vivo* experimental models of HI brain injury. It could augment B-cell lymphoma 2 (Bcl-2) and brain-derived neurotrophic factor (BDNF) in asphyctic rats [19, 118]. In addition, a phase 1 study reported that

**Table 1. Experimental models for HI.**

| Refs. | Species | Animal Model | Outcomes |
|---|---|---|---|
| **Large Animal Models** | | | |
| [50] | Macaca nemestrina, near term | UCO | Poor weight gain and cerebellar growth, abnormal brain DTI, behavioral impairment, 43% develop CP |
| [58, 59] | Fetal sheep, near term | Bilateral CCAO | Shorter HI (<30 min): selective neuronal loss. Longer HI: cortical necrosis. Post-HI EEG suppression related to insult severity and pathology; prevented by hypothermia |
| [47] | Fetal sheep, midgestation | Bilateral CCAO | Necrosis of subcortical white matter, neuronal loss in thalamus and striatum similar to near term fetus. Little loss of final EEG amplitude |
| [60] | Fetal sheep, midgestation and near Term | UCO | Hippocampal neuronal loss only in near term group. Degree of injury associated with the severity of hypotension during UCO |
| [61] | Pigs, <24h old | CCAO + hypoxia | Secondary energy failure. Energy metabolism ameliorated by hypothermia (35°C for 12h) at 24h-48h |
| [62] | Pigs, P9 | Hypotension + hypoxia | ~60% fall in CBF, reduced cerebral $O_2$ uptake, phosphorylated metabolites and pH and increased inorganic phosphate |
| [44] | Rabbits, 21–22 d gestation | Uterine ischemia | P1 pups: overt posture and tone after ischemia > 37 min, correlates with microgliosis in basal ganglia and thalamus. MRI: WMI in IC |
| **Rodent Models with Global Hypoxic or Excitotoxic Component** | | | |
| [63] | Mice at E8, P0 or P5 | Ibotenate, i.c.v. | laminar neuronal depopulation of layer V–VIa. P5: neuronal loss in all cortical layers, formation of porencephalic cysts |
| [64] | Pregnant Sprague-Dawley rats, embryonic | Hypoxia E5-E20 | White matter cysts in offspring P0–P7, increased lipid peroxidation, WMI and macrophages |
| **Rodent Models with Hypoxia-Ischemia** | | | |
| [65, 66] | Sprague Dawley rats, P1–P3 | CCAL + hypoxia | Selective vulnerability of late OL progenitors, independent of age<br>Death of sub-plate neurons, motor deficits, altered thalamocortical connections to somatosensory and visual cortex normal |
| [43] | Sprague-Dawley rats, P7 | CCAL + hypoxia | Unilateral ischemic injury in the cortex, hippocampus, basal ganglia in > 90% of survivors |
| [67] | Wistar rat, P7 | LPS, 4h prior to CCAL + hypoxia | Blocking lymphocyte trafficking reduced brain inflammation, BBB damage, and improved LPS-induced HI brain injury. No effect with pure HI |
| [68] | C57Bl/6 WT, Tg SOD1, GPx1 over-expressing P7 mice | CCAL + hypoxia | Reduced injury in GPx1-Tg mice but not in SOD1-Tg or GPx1/SOD1. NOS inhibition did not improve outcome in SOD-Tg |
| [69, 70] | C57BL/6 WT and Gal-3 KO, P9 | CCAL + hypoxia | Increased BBB permeability 2–24h, reduced BBB protein expression. Infarct volume reduction in Gal-3 KO mice |
| [71] | C57BL/6J and TRIF KO mice, P8–9 | Poly I:C, 14h prior to CCAL + hypoxia | Increased infarct volume and WMI, prevented in TRIF KO. Injury linked to inflammatory response & decrease in M2-like microglia |

**(Table 1) contd….**

| Refs. | Species | Animal Model | Outcomes |
|---|---|---|---|
| **Focal Ischemia Rodent Models** | | | |
| [72] | Wistar rat, P7 | Permanent MCAO + 1h CCAO | Infarcts in frontoparietal cortex at 3-month recovery. DNA fragmentation from 6–96h |
| [73-75] | Sprague Dawley rats, P7 | Transient MCAO, 3h | Severe unilateral perfusion deficits, restoration of CBF upon suture removal. Decreased ADC associated with brain injury at 24h reperfusion. Demonstrated endogenous neuroprotective role of microglial cells after acute injury |
| [76] | Sprague Dawley rats, P10 | Transient MCAO, 1.5h | Time resolved cell-type specific increase in HIF-1α and VEGF expression, gliosis |
| [77] | C57/Bl6 mice, CD36 KO and WT, P9 | Transient MCAO, 1.5h and 3h | Focal ischemia-reperfusion, increased injury and caspase-3 cleavage associated with apoptotic neuronal debris in CD36 KO. Effects independent of NFκB activation |
| ***In Vitro* Models** | | | |
| **Refs.** | **Cell Line** | **Experimental Model** | **Outcomes** |
| [78] | PC12 cells | 48 h OGD/ 2h reperfusion | Significant morphological cell changes |
| [79] | Primary cortical astrocyte | 6 h OGD/ 0, 12, 24, 48 h reperfusion | Significantly increased 2- NBDG uptake by about 1.2 to 2.5 times in cells compared to control |
| [80] | Primary cerebral cortex neurons | 3 h OGD/ 48 h Reperfusion | Damage to neuronal viability, dendrite branch number in neurons deceased significantly |
| [81] | Primary astrocyte | 3, 5, 7 h OGD/ 24 h Reoxy-genation | Increases in HMGB1 and TNF-a, induced phosphorylation of PI3K, pro-moted nuclear translocation of NF-kB |
| [82] | primary cortical neurons | 2 h OGD | Suppressed significantly cortical neurons proliferation |
| [83] | SH-SY-5Y cells | 6 h OGD/ 1h reoxygenation | Caused significant mitochondrial fragmentation, excessive mitochondrial fission |
| [84] | Primary Cortical Neuron | OGD | Decrease in neurite outgrowth |
| [85] | Neural progenitor cell | 6 h OGD | Increased apoptosis |
| [86] | mouse hippocampal neurons HT22 | 4 h OGD/ 24 h Reoxygena-tion | miR-144-3p expression was significantly downregulated in neurons following OGD/R treatment |
| [87] | Neuro 2a cells | 4 h OGD/ 12 h Reoxygena-tion | Inhibited cell viability and cell proliferation, reduced phosphorylation levels of p38 MAPK and ERK1/2 |
| [88] | SH-SY5Y cells and primary murine cortical neurons | 4 h OGD | OGDR-induced mitochondrial depolarization, reactive oxygen species production, lipid peroxidation and DNA damages |
| [89] | Primary astrocytes and microglial cells | 2 h OGD/ 48 h Reoxygena-tion | Induced abnormally opened hemichannels with increased ATP release and EtBr uptake but reduced GJIC permeability<br>Astrocytic Cx43, hemichannels, and GJIC play critical roles in OGD/R injury-induced neuroinflammatory responses |
| [90] | Primary astrocytes | 4 h OGD/ 3 h, 6 h, 12 h, 24h reoxygenation | Expression of Ski was proved to be up-regulated |
| [91] | Primary hippocampal neurons | 2 h OGD/ 24 h reperfusion | Caspase-3 activity and expression increased in the first 24 h |
| **HMAs Models** | | | |
| **Refs.** | **Cell Line/species** | **Experimental Model** | **Outcomes** |
| [92] | multiple myeloma cell line U266 | $CoCl_2$ | $CoCl_2$-mediated hypoxia affects the expression profiles of genes that are functionally related to apoptosis and angiogenesis |
| [93] | myeloid leukemic cell lines NB4 and U937 | $CoCl_2$ and DFO | Apoptosis with a loss of mitochondrial transmembrane potentials, activa-tion of caspase-3/8 and cleavage of anti-apoptotic protein Mcl-1 |
| [94] | U251 human glioblastoma cell line | $CoCl_2$ | Increases HIF-1a gene expression |

**(Table 1) contd….**

| Refs. | Cell Line/species | Experimental Model | Outcomes |
|---|---|---|---|
| [95] | glioblastoma cell lines U373MG and DBTRG05MG | DFO | Activation of factors associated with ECM degradation and invasion of glioma cells |
| [96] | C57BL/6 mice | DFO | DFO up-regulated the expression of vascular endothelial growth factor (VEGF), HIF-1α protein and growth associated protein 43 (GAP43) and down-regulated the expression of divalent metal transporter with iron-responsive element (DMT1+IRE), α-synuclein, and transferrin receptor (TFR) |
| [97] | Hippocampal neurons | DFO pretreatment/3h OGD | 45% reduction in cell death |
| [98] | Sprague-Dawley rats | subarachnoid hemorrhage/DFO treatment | DFO-induced increase in HIF-1 protein level and activity exerts significant attenuation of BA vasospasm |
| [99] | Hippocampal cultures | Ppreconditioning $CoCl_2$, DFO or dimethyloxylalyglycine (DMOG), 3h OGD | Cobalt induced the transcription of the cytokine erythropoietin. cobalt and DFO, enhanced survival of neurons. DMOG exacerbates OGD-induced neuronal death |
| [100] | Sprague-Dawley rats | CCA/DFO treatment | Neural-protective and angiogenesis effects through regulating the levels of HIF-1α |
| [101] | adipose-derived stem cells | DFO preconditioning | Restored neovascularization potential of ADSCs |
| [102] | Sprague – Dawley rats | MCA/DFO treatment | Preserved brain volumes, upregulation of HIF1α |
| [103] | Wistar rats | MCAO/DFO+Erythropoietin treatment | Reduced the number of cleaved caspase 3-positive cells in the ipsilateral cerebral cortex. |
| [104] | Human mesenchymal stem cells | $CoCl_2$ and DFO | Proliferation of hMSCs was inhibited by DFO and CoCl |

Modified from [105].



breathing 50% of xenon for 18h didn't show adverse effects for newborns even after 18 months follow-up. Nevertheless, the high-cost and difficulty in the administration of xenon are the main barriers for its clinical use [19].

Melatonin was found to be protective in MCAO at post-natal day 12 (P12). This effect was probably mediated by inhibition of autophagy and neuronal cell death as well as enhanced Growth Associated Protein 43 (GAP43) expression [119]. Erythropoietin (EPO) is a naturally occurring hormone produced with angiogenic action. It presents neuroprotective, neurogenic, anti-inflammatory and anti-apoptotic properties [20]. In a rodent model of adult stroke, rhEPO increased vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF), reducing the size of stroke infarct and improving functional outcomes [20].

A novel drug, orientin, is under study in a rat cortical neurons primary culture model of OGD. This molecule is a water-soluble flavonoid *C*-glycoside, commonly extractable from different plants like the passion fruit with antioxidant properties. The pretreatment dose-dependently inhibited OGD/Reperfusion (RP) induced phosphorylation of c-Jun N-terminal kinases (JNK) and Extracellular signal–regulated kinases (ERK)1/2. In addition, JNK inhibitor SP600125 and ERK1/2 inhibitor PD98059 ameliorated OGD/RP-induced cell death and ROS generation. Further, orientin failed to protect cortical neurons with the JNK activator anisomycin or with the ERK1/2 activator FGF-2 [6], showing that the neuroprotective effects of orientin against OGD/RP-induced damage depend on down-regulation of JNK and ERK1/2 signaling pathways [6]. In a model of OGD/R injury in a primary co-culture of rat cortical capillary endothelial cells, astrocytes and neurons to allow simultaneous observations in the same system, it was found that pretreatment with Ginkgo biloba extract or Ginkgolide B increased neuronal viability, ameliorated cell injury, and inhibited cellular apoptotic rate through Bcl-2 and Bcl-2-associated X protein (Bax) expression [7]. On the other hand, allopurinol, a xanthine oxidase inhibitor and free radical scavenger, reduced cerebral edema and brain damage induced by perinatal HI in P7 rat pups. Nonetheless, a meta-analysis of three clinical trials showed no significant benefits for neonates with HI encephalopathy treated with allopurinol [19].

Stem cells have been used in clinical trials to treat stroke on adults. Keeping in mind that stroke in adults and HI in neonates share many symptoms, neuroprotective treatments for stroke might also be effective in neonates [120]. Umbilical Cord Blood Cells (UCBs) have shown anti-inflammatory and anti-apoptotic properties in animal models of HI encephalopathy. UCBs are composed of different cells such as lymphocytes, endothelial progenitors, hematopoietic stem cells, monocytes and mesenchymal stem cells (MSCs) [20]. Following an injury, MSCs promote neuronal differentiation, augment cellular regeneration and reduce immune cell response [20].

Norm baric hyperoxia (NBO) has been shown to exhibit neuro- and vaso-protective effects by improving tissue oxygenation when it is given during ischemia. However, the effect of NBO might diminish when the duration of ischemia and reperfusion is extended. For that reason, it should be

administered together with drugs that could enhance the protective effects [121].

### 4.1. Therapeutic Hypothermia

Currently, the only available treatment for PA is therapeutic hypothermia (TH) [20]. TH is the unique treatment available after resuscitation from moderate- to-severe perinatal HI. It should be started as soon as possible, at least within 6 h after birth, and continued for 72 h [18]. Using TH before the onset of the secondary energy failure prevents the development of cytotoxic edema, cell death and brain damage [18, 122].

It has been shown that mild hypothermia could reduce or inhibit secondary impaired oxidative phosphorylation, augmenting ATP and correcting Protein C reactive (PCr) levels after HI [18]. TH protects the brain by reducing brain metabolism in almost 5% for every degree of temperature lowering [122]. Reducing cerebral metabolism provokes the attenuation of oxidative stress, excitotoxicity, inflammation, cell death pathways and the accumulation of essential amino acids due to the delay in the depolarization [18, 122]. The efficacy of TH can be clinically and experimentally tested with the use of prognostic 1H magnetic resonance spectroscopy (MRS) biomarkers [18].

Immediate use of TH after HI reduces caspase-3 expression in oligodendrocytes and cortical infarct at P7, an age when murine brain development presents similarities with the late preterm human infant [122]. By the same way, TH has shown reduction of apoptosis in neurons after 24 hours, decreasing the activation of caspases-3, -8, and, -9, and the migration of cyt-c [122]. TH seems to have beneficial effects on astrocytes as well. In a neonatal-rat brain slice OGD model, an augment of glutamine was observed in the TH group. This change was registered after the injection of a mixture of glucose and acetate, which converts glucose into glutamine *via* the pyruvate carboxylase, and is related only to astrocytes metabolism [18, 123]. Similarly, intra ischemic hypothermia maintains mitochondrial activity in neonatal rats after 4 days of recovery. Nevertheless, it is unclear if TH directly protects the mitochondria or if this effect depends on the suppression of apoptosis and inflammation [122, 124].

Finally, a meta-analysis of randomized clinical trials showed that TH is beneficial for term infants with moderate-to-severe HI encephalopathy, producing a relative risk reduction of mortality or neurodevelopmental disabilities [18, 125]. However, mild hypothermia treatment after intravenous thrombolysis in patients with acute stroke did not show protective effects [126, 127]. Therefore, it becomes necessary to find drugs that enhance the efficacy of mild hypothermia [18, 127].

### 4.2. The Neuroprotective Role of 17β-estradiol in HI

Neuroactive steroids (NASs) are naturally or artificially synthesized steroids which exert actions on the brain. Most of the naturally synthetized steroids are made as *de novo* from cholesterol [128, 129]. NASs are classified as pregnane, androstane, and sulfated neurosteroids (NSs) [129]. They regulate different processes interacting with: gamma-aminobutyric acid (GABA), microtubule-associated protein 2 (MAP-2), NMDA, serotonin, α-adrenoreceptors, transient receptor potential channels, voltage-gated calcium channels, voltage-dependent anion channels, neurotrophins, nerve growth factor, and σ1 receptors [129]. Other molecules, such as synthetic steroid analogues, are used for exerting estrogenic effects with major stability. Several studies have shown 3α-hydroxyl configuration is the key for binding and activating these molecules, but modifications in the steroid nucleus may emphasize different pharmacophores [130].

A derivate of estrogen, 17β-estradiol, is a female sex hormone and NAS related to the development of secondary sexual characteristics, fat storage and regulation of menstrual cycle [131]. 17β-estradiol, which was originally administered as a hormone replacement therapy in order to ameliorate climacteric symptoms, showed beneficial effects in verbal and visual memory performance [132]. The activity of 17β-estradiol depends on its union with estrogen receptors (ERs) [35, 132, 133]. These receptors are classified in two subtypes: the estrogen receptor-alpha (ER-α) and the estrogen receptor-beta (ER-β). Locus from ER-α and ER-β are chromosome 6 and chromosome 14, respectively [132]. These ERs are transcription factors which present the peculiarity of being activated by a ligand. ER-α and ER-β have a similar structure, with a DNA-binding domain and a ligand-binding domain [134]. 17β-estradiol binds to ERs and induces the activation and the homodimerization or heterodimerization of these receptors. Then, the ERs bind to estrogen-responsive elements (EREs) in the promoter region of specific genes through the DNA-binding domain, recruiting transcriptional co-activators and co-repressors [134, 135]. Classical ERs may also regulate gene transcription by acting as transcriptional partners at non-ERE sites, such as activating protein 1 (AP1) sites [136]. 17β-estradiol can bind to membrane-associated non-classical ERs, such as G protein-coupled ERs (GPERs). GPER30, a member of the G protein-coupled receptor superfamily, regulates the activity of extracellular signal-regulated kinases (ERKs) and the phosphoinositide 3-kinase (PI3K) signaling pathway. This union allows the interaction with the signaling of other neuroprotective molecules [134, 137]. Another membrane-associated non-classical ER is Gαq protein-coupled membrane ER (Gq-mER), which was originally identified in hypothalamic neurons, modulating μ-opioid and GABA neurotransmission [134, 138].

Estrogen and its derivatives may exert their activity through genomic and non-genomic responses [134]. However, these responses might be overlapped [139]. Genomic responses require the translocation to the nucleus, binding to EREs and transcription factors in order to achieve a long-lasting response [140, 141]. Estrogenic non-genomic response involves signaling mechanisms that allow estrogen to accomplish rapid and dramatic effects [142]. These responses act through a second messenger, generating several signal transduction cascades, such as ion fluxes (specially calcium), cyclic AMP modulation and protein kinase pathways, giving an acute response between seconds or minutes, mainly through P13K or MAPK pathways [140, 143].

In a murine model of memory consolidation, 17β-estradiol enhanced hippocampal memory *via* rapid activation of Erk MAPK and PI3K/Akt [144, 145], suggesting that 17β-

estradiol could increase spinogenesis in hippocampus and learning experience through kinase cascades [146] Tozzi *et al*. (2015) evaluated the interactions between ERs and dopamine (DA) receptors in dorsal striatum of adult male rats, showing that inhibition of 17β-estradiol synthesis by aromatase P-450 inhibition prevented the induction of long-term potentiation (LTP) in both medium spiny neurons (MSNs) and cholinergic interneurons (ChIs). Downstream estrogen signaling pathway activated D1-like DA receptor/cAMP/Protein kinase A (PKA)-dependent pathway, restoring LTP, and exogenous 17β-estradiol administration reversed the effect of the aromatase P-450 inhibition., confirming 17β-estradiol is involved in LTP of MSNs [147].

Non-genomic actions of estrogens in different cell contexts usually are mediated by GPR30, functionally cross-reacting with diverse cell signaling systems such as the epidermal growth factor receptor (EGFR) pathway, Notch and mitogen-activated protein kinases (MAPK) signaling pathways [148]. 17β-estradiol exerts neuroprotective effects against ischemia by activation of GPR30, which is linked to transactivation of Insuline-like growth factor 1 (IGF-1) receptor [149]. 17β-estradiol increases the expression of basic fibroblast growth factor (bFGF) in astrocytes [150], inducing neuroprotection against ischemia and glutamate-induced excitotoxic neuronal cell death [151-153]. These findings have led to research on the neuroprotective properties of estrogen and its derivates on brain injury.

In HI brain injury 17β- estradiol has shown several neuroprotective effects, such as: reducing reactive gliosis, decreasing oxidative stress, ameliorating the release of pro inflammatory molecules, preventing cell death and mitochondrial dysfunction, releasing neurotrophic factors [154]. It has also been reported that 17β- estradiol may exert protective actions against OGD-induced cell death in primary oligodendrocytes. In this way, 17β- estradiol presents neuroprotective properties in hypoxic-ischemic and oxidative injury [155].

A repeated dosing paradigm (three doses of 17β-estradiol) in neonates subjected to HI provided approximately 70% protection in the hippocampus, basal ganglia, and amygdala [156]. In addition, treatment with estradiol after PA augmented the expression of IGF-1 and its receptor (IGF-IR). The PI3K/Akt/GSK3 signaling pathway was activated as an increase in Akt and GSK3 phosphorylation [157]. However, it has been found that male sex is a well-established epidemiological risk factor for poor neurodevelopmental outcome after PA. While the mechanisms responsible for this gender difference are unknown, growing evidence has identified neuro-inflammation, oxidative stress and cell death pathways as key players in these differences [158].

Using a mice model of MCAO with a mutant form of ER-α, neuroprotection was absent, showing that protective properties depend on Er-α [159]. Similarly, after emulating hypoxia in the neuroblastoma cell line SH-SY5Y by using $CoCl_2$ (250 μg/mL), a hypoxic mimetic agent, treatment with 17β-estradiol (250 nM) exerted neuroprotection. Afterwards, using ER-α and ER-β agonist (PPT and DPN, respectively) without 17β-estradiol treatment, results showed neuroprotection was mimicked by PPT and suggested that ER-α regulates this protective effect [157].

Likewise, in a model of astrocytic cells it was found that treatment with estradiol improved HI parameters such as cell viability and mitochondrial membrane potential, besides reducing ROS production and preventing the loss of mitochondrial mass [160]. Nevertheless, estrogen might have detrimental effects, such as the augmentation in breast and uterus cancer incidence [10-12]. In order to maintain the benefits of estrogen and avoid side effects, synthetic steroid analogues have been developed. These group of drugs includes SERMs, such as raloxifene and tamoxifene, and STEARs, like tibolone [10-12].

The mechanism of regulation of SERMs that determines if they act either as agonist or antagonist in a specific cell type depends on the predominant subtype of estrogen receptor α or β. The regions of union to the ERs are: activation function 1 (AF-1), capable of binding with the DNA, and activation function 2 (AF-2), a region of union with the ligand in order to achieve an estrogenic response. The ligand must bind to the AF-2 region of the ER and migrate to the nucleus to bind the response site. Co-activators, co- factors and helper proteins of each cell will determine the kind of response of the tissue exposed to SERMs [161, 162].

In rats subjected to MCAO and treated with estrogen and Raloxifene, neurogenesis in the ipsilateral subventricular zone (SVZ) after ischemia was significantly higher in estrogen and raloxifene-treated animals compared to rats treated with placebo. Tamoxifen did not show this enhancing effect on neurogenesis. However, both tamoxifen and raloxifene as well as estrogen, significantly reversed spine density loss observed in the ischemic cortex at day-5 post ischemia [163].

Tibolone action is given by the metabolization of tibolone to three different metabolites (δ-4 tibolone, α -hydroxy tibolone and 3-β-hydroxy tibolone). Each of them produces different responses. δ -4 tibolone is an agonist of androgen and progesterone receptor, while α-hidroxy and β-hidroxy tibolone are antagonists of those receptors but agonists of ERs [164]. Keeping this in mind, it was found tibolone could ameliorate the effects of GD in an *in vitro* model of astrocytes, making these molecules interesting for further research in an OGD model [11].

## 5. COMBINED THERAPIES

### 5.1. Combined Therapies Using TH

The lack of effective treatments against HI and the little success of clinical trials for different treatments, lead to a search for new and better therapies. Since TH is the most widespread therapy against the deleterious effects of HI injury, it has become the basis for the development of combined therapies or co-treatments [20]. Combined treatments aim to use the protective effects of each of its components (Table **2**). TH can prevent the development of brain edema, as well as reducing the damage produced by inflammation, oxidative stress and excitotoxicity [18]. Similarly, TH has also shown the ability to improve mitochondrial autophagy [106] and prevent apoptosis by preventing the translocation of the apoptosis-inducing factor (AIF) to the nucleus [165].

**Table 2. Summary of combined therapies.**

| Refs. | Therapy | Model | Outcomes |
|---|---|---|---|
| [178] | Erythropoietin+ Hypothermia | Randomized trials in infants | preterm infants assigned to receive rhEPO showed that the rate of moderate/severe neurological disability in the rhEPO group was (7.1%)significantly lower compared to the placebo group (18.8%; $p < 0.001$) |
| [179] | Melatonin + Hypothermia | Piglet model of HI | Improved white matter tract development and reduced apoptosis |
| [166] | Xenon + Hypothermia | Asphyxiated Piglets | Xenon with hypothermia, offers histopathological and functional neuroprotection |
| [167] | Mesenchimal stromal cells + Hypothermia | Primary Neurons from rats | shown neuroprotection in rat primary neurons preserving cell viability |
| [127] | Mild hypothermia + glibenclamide; dizocilpine; neuroglobin | Primary cortical Neurons | co-treatment of those three drugs and mild hypothermia decreased ROS and intracellular calcium accumulation and stabilized mitochondrial membrane potential (MMP) |
| [177] | 17β-estradiol + Hypothermia | Rats with HI | low dose of 17β-estradiol after the ischemic event exerted neuroprotective effects for the transient global ischemia, and its effect is potentiated by therapeutic hypothermia |
| [180] | CIMT + EA | Rats with HI | -CIMT combined with EA significantly reduced motor asymmetry after Hi. augmented NeuN and reduced GFAP expression in the cortex |
| [181] | Minociclyne + NBO | Rats on MCAO | Neuro- and vaso-protective effects by inhibiting matrix metallo-proteinsase (MMP)-2/9-mediated occludin degradation and attenuation of caspase-dependent and independent apoptotic pathways |
| [182] | Ederavone + NBO | Mice HI reperfusion | Combined treatment reduced the infarct zone volume, getting better neurological functions, in cortex and subcortex after 22 hours of reperfusion |
| [183] | Cilostazol + NBO | Mice with HI | the combined therapy ameliorated the damage done by focal cerebral ischemia/reperfusion injury, by enhancing the blood flow to the damaged area after the reperfusion, and this effect was related to eNOS activity |



Some combined treatments approved for clinical trials involve EPO combined with hypothermia. Neonates with PA treated with a combination of rhEPO and hypothermia showed improved neurological outcomes, as well as fewer white matter tract abnormalities. Melatonin combined with clinical hypothermia improved white matter tract development and reduced apoptosis in a piglet model of HI [20].

Xenon, a noble gas that exerts neuroprotective effect by up-regulating Bcl-2 pro-survival proteins [18], offers histopathological and functional neuroprotection in combination with hypothermia [166]. Another combined therapy that has shown promising results in rat primary neurons is the treatment with mesenchymal stromal Cells and hypothermia, which preserves cell viability [167]. Finally, a recent study evaluated the neuroprotective activity of 26 different medicines with neuroprotective potential in combination with TH in cortical neurons subjected to OGD, finding a significant neuroprotective effect using BDNF, glibenclamide (GBC), kallidinogenase (HUK), (1) -MK-801 ((1) -Dizocilpine hydrogen maleate (MK-801) and neuroglobin (Ngb).

Taking into account the few options of combined treatments to date, it is imperative to deepen the search for new therapies that help combat the negative effects caused by HI. SERMs and STEARs represent a promising option in conjunction with hypothermia. As for SERMs, in murine models of MCAO raloxifene increases neurogenesis in the ipsilateral subventricular zone, and tamoxifen together with raloxifene and estrogen reverse the loss of spine density [163]. Similarly, raloxifene has shown neuroprotective G protein-coupled receptor 30 (GPR30) -dependent activity in an OGD model of rat neuronal cells [168]. Likewise, raloxifene exerted neuroprotective effects on neocortical cells in a caspase-3-independent manner [169, 170]. In addition, raloxifene and tamoxifen have the ability to reduce microglial activation in rats after HI brain injury [171].

With regards to STEARs, tibolone may exert neuroprotective effects in cerebellum and hippocampus, reducing tau hyper phosphorylation and modulating Glycogen synthase kinase-3b (GSK3b) phosphorylation [172]. Additionally, tibolone has shown a protective effect against oxidative stress and lipid peroxidation [173, 174]. It also has the ability to modulate reactive gliosis and reduce the number of microglial cells with a reactive phenotype, exerting beneficial effects on the homeostasis of cerebral cortex [175]. This STEAR is also capable of modulating the expression of Ngb in astrocytic cells in a model of GD [176].

Keeping in mind the neuroprotective effects exerted by the SERMs and STEARs, it is possible to suggest that the multifactorial neuroprotective action of TH may act synergistically in a possible combination treatment with these compounds, since they also exert their beneficial action from various points of action. Although research on the neuropro-

tective effect of TH combined with SERMs or STEARs is missing, combination treatment with 17β-estradiol and TH has shown greater efficacy than monotherapies [177], suggesting NASs might potentiate the protective effects of TH. The distinctive but complementary protective mechanisms of NASs and TH might be responsible for the synergistic effect of a combined therapy [177].

### 5.2. Other Combined Therapies

Other combined therapies (Table **2**) do not include TH but have not been approved for clinical trials yet. A combined therapy of constraint-induced movement therapy (CIMT) and electroacupuncture (EA) was tested in neonatal rats with HI-induced hemiplegia. CIMT consists in the inhibition of the normal limb, forcing the animals to use the damaged one. EA is based on using needles in acupuncture points and applying electrical stimulation. This combined therapy has shown effective results in stroke rehabilitation and hemiplegia [180]. CIMT combined with EA reduced motor asymmetry after HI, but HI lesion size was not improved. This treatment augmented NeuN and reduced GFAP expression in the cortex, suggesting it targets both neurons and astrocytes. In addition, CIMT combined with EA reduced the expression of cleaved caspase-3, an apoptotic mediator [180].

Combined therapy using NBO and minocycline provided greater neuro- and vaso-protective effects than monotherapy, by inhibiting matrix metallo-proteinsase (MMP)-2/9-mediated occludin degradation and attenuating caspase-dependent and independent apoptotic pathways [121,181]. NBO combined with ederavone, a scavenger of hydroxyl radicals, exerted greater effects that monotherapies by reducing the infarct zone volume and improving neurological functions in cortex and subcortex after 22 hours reperfusion [121,182]. Cilostazol combined with NBO has been reported to protect the brain after cerebral ischemia in rodents, ameliorating the damage caused by focal cerebral ischemia/reperfusion injury. This effect was associated with an enhancement of blood flow to the damaged area after reperfusion, which is related to eNOS activity [121,183].

Tissue plasminogen activator (tPA) can solve blood clots to restore blood flow in the ischemic brain region. Until now, the administration of tPA within 3 or 4.5 hours after symptom onset is the only FDA approved treatment for acute ischemic stroke. However, its combination with NBO has the potential to expand the therapeutic time window for tPA administration and to reduce subsequent reperfusion induced injury [121]. Similarly, combination therapy of Umbilical cord blood cells (UCBs) and EPO resulted in significant improvements in motor and cognition, with associated improvements in structural and metabolic changes in the brain [20].

## CONCLUSION

HI events exhibit a high impact on society. Over the years, research has intended to find effective treatments that counteract the damage caused by HI. Neuroprotective targets include specific points of damage caused by HI, such as oxidative stress, dysregulation of the cell cycle and energy homeostasis [20]. Both in the initial damage phase and in the final one, the different neuroprotective agents may exert anti-inflammatory, antioxidant, anti-excitotoxicity or anti-apoptotic effects [19]. However, due to the complex network of factors that influence these pathologies, including cellular interactions inherent to the CNS as well as gender-dependent response [158], the effectiveness of neuroprotective treatments has not been optimal.

Several studies have focused on TH, which with its multifactorial action represents the only clinical treatment available for human use. However, TH has shown a relative success. Bearing this in mind, research has currently focused on the search for new neuroprotective agents that carry out their activity from different fronts, whereupon SERMs and STEARS arise as an alternative [10-12]. *In vitro* and *in vivo* studies should be developed to give a better understanding of the processes involved for a better implementation of the treatments.

Following this logic, the search for treatments that not only focus their attention on improving a specific parameter seem to be a better alternative. Combined treatments or co-treatments respond to this need. These assume TH as a therapeutic basis by combining it with different drugs [13]. Investigations of the mechanisms by which this combination therapy exerts its neuroprotection are currently taking place. Likewise, SERMs and STEARs emerge as promising candidates in the implementation of a combination therapy due to their benefits in different parameters. Further research is necessary for proving this hypothesis.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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