Neuroprotective effect of antioxidants in neonatal rat brain after hypoxia-ischemia

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Perinatal hypoxic-ischemic (HI) encephalopathy is one of the main causes of mortality and long-term disabilities in newborns. The adverse effect of birth asphyxia (deprivation of oxygen and glucose) in the brain can be devastating, resulting in death or severe neurological consequences such as mental retardation, learning disabilities, epilepsy and cerebral palsy. In spite of the advances in neonatology, The World Health Organization estimates that 4 to 9 million neonates suffer from birth asphyxia each year in the world, thus, the identification of new treatments is urgently needed. Nowadays, there are experimental evidences demonstrating that some antioxidant compound are effective in different animal models of HI brain injury, being able to reduce infarct volume and neuronal loss. In this review we will focus on the neuroprotective effect of resveratrol (RVT) and docosahexaenoic acid (DHA) in experimental HI encephalopathy and also discuss the possible synergy between different antioxidant agents.

Keywords: Hypoxia-ischemia; antioxidants; neuroprotection; resveratrol; docosahexaenoic acid

1. Introduction

Neonatal hypoxia-ischemia and subsequent brain damage remains still as an important socio-sanitary problem, in spite of the advances in obstetric and neonatal care over the last decades. Perinatal asphyxia occurs in 1-10 of every 1000 live term births [1] and is considered the single most important cause of acute mortality and chronic disability in newborns worldwide [2,3]. Perinatal asphyxia may occur both prenatal (during birth and delivery) or in the immediate postnatal period. Data from epidemiological and neuropathological studies have identified several maternal and fetal risk factors, including placental abruption, cord compression, transplacental anesthetic or narcotic administration, intrauterine pneumonia, severe meconium aspiration, congenital cardiac or pulmonary anomalies, birth trauma, obstructed airway, maternal opiated and congenital sepsis [1].

The result of a deprivation of oxygen and glucose to the brain give rise to death or severe neurological consequences such as cerebral palsy, mental retardation, visual and hearing impairment, learning and behavioral disabilities, attention deficits and hyperactivity and epilepsy [4-6]. The severity of neonatal encephalopathy depends on the intensity, duration and location of the insult [7]. The 15-20% of affected newborns will die in the postnatal period and an additional 25% will develop severe and permanent neuropsychological sequelae [8] with only a small percentage of infants with severe injury will survive without any handicap [9].

The clinical diagnosis of neonatal hypoxia-ischemia is based on two different criteria, one of them consists of evidence of neurological and cardio-respiratory depression (obtaining less than 7 in the Apgar score at 5 minutes after birth) and the other one consists of acidemia (defined as an arterial blood pH of less than 7), as the term asphyxia is defined experimentally as impaired respiratory gas exchange accompanied by the development of metabolic acidosis [10].

2. HI injury

The brain is the most metabolically active organ in the body, about the 95% of the consumed oxygen is reduced to ATP and the remaining 5% is released as radical oxygen species (ROS). The brain has a relatively high content of membrane lipids susceptible to oxidation and low antioxidant defense. Therefore, brain tissue maintains a fragile redox homeostasis and neurons are especially vulnerable to free radical damage. Under physiological conditions the antioxidant enzyme system balances redox homeostasis avoiding cell damage, but under ischemic conditions this system fails to protect cells from injury [11].

The developing brain is more susceptible than the adult one to HI events [12,13], due to its high concentration of unsaturated fatty acids, high rate of oxygen consumption, low concentration of antioxidants (vitamin E and ascorbic acid), high water content, low myelinization, availability of redox-active iron, an imbalance of antioxidant enzymes (catalase, Cu/Zn-superoxide dismutase-1 (SOD-1), mitochondrial superoxide dismutase-2 (SOD-2) and glutathione peroxidase (GPx)), and oxygen-induced vasoconstriction [7,14-17].
Insult from hypoxia-ischemia causes immediate neuronal injury and exhaustion of cellular energy stores, as the main cause of HI brain injury is the deprivation of glucose and oxygen supply, which initiates a multi-faceted cascade of biochemical events, as showed in Figure 1. Schematically two metabolic phases are recognized in the neurological damage, the first one is the primary energy failure due to the HI event and the second phase is consequence of the reoxygenation taking place some hours later.

Primary energy failure induces a decrease in oxidative phosphorylation and results in a change to anaerobic metabolism in order to maintain basic functions. Anaerobic metabolism, which is energetically inefficient, leads to a rapid depletion of ATP, accumulation of lactic acid and failure of ion pumps, resulting in a massive entry of sodium, calcium and water into the cells. The membrane depolarization results in an excessive release of excitatory neurotransmitters, mainly glutamate which activates N-methyl-D-aspartate (NMDA) receptors triggering excitatory cascade and promoting death [18]. The secondary phase of damage, which occurs from 6 to 48 hours after the primary event, is related to the reoxygenation, involving a mitochondrial dysfunction that extends and increases the reactions from primary phase [19]. The massive increase in free cytosolic calcium concentration induces the production of nitric oxide (NO) by activation of neuronal nitric oxide synthase (nNOS) and the generation of free radicals (as we will see below in section describing oxidative stress). This results in the degradation of cellular lipids (by activation of phospholipases), proteins (by activation of proteases), and DNA (by activation of nucleases) [20-26].

The combined effects of cellular energy failure, acidosis, glutamate release, intracellular calcium accumulation, lipid peroxidation, and nitric oxide neurotoxicity provoke, in many cases, the death of the cells, either by necrosis or apoptosis [27,28], a divergence that will depend on the severity of the insult, the maturational state of the cell or the brain region affected, among others. Necrosis usually occurs in regions with more damage, while apoptosis appears in regions with moderate injury [32]. On the other hand, the first usually appears immediately after the insult, while the later can persist for at least 7 days after injury [29-31]. Mitochondria play a central role in the apoptotic process, as the permeabilization of its outer membrane can trigger the release of the apoptosis-inducing factor and the cytochrome C, which leads to the cleavage of caspases 9 and 3, two of the major effectors of delayed cell death [33].

3. Oxidative stress

Oxidative stress (OS), which is defined as an imbalance between oxidant and antioxidant factors, contributes to neural cell damage following HI brain injury. There is little doubt about the fact that OS is a major contributor to ischemic brain injury [34], because it is an important consequence of the neurotransmitter-mediated toxicity following perinatal
As we have seen the brain is highly at risk of free radical-mediated injury because neuronal membranes are rich in polyunsaturated fatty acids and because the human newborn has a relative deficiency in brain SOD and GPx. Since antioxidant defense system do not accelerate in maturation until late third trimester of pregnancy [45], exogenous antioxidant therapy would be helpful in order to prevent cellular damage if perinatal asphyxia occurs. In this review we will focus on the neuroprotective effect of resveratrol and docosahexaenoic acid in experimental HI encephalopathy and also discuss the possible synergy between different antioxidant agents.
Fig. 2 Representative light microphotographs of hematoxylin-eosin stained brain sections obtained from dentate gyrus in the hippocampus of neonatal rats. A) Control; B) HI rat; C) HI rat with RVT; and D) HI rat with DHA. Bar: 40 µm.

4.1 Resveratrol

Resveratrol (3,5,4′-trihydroxystilbene) is a non-flavonoid polyphenolic compound consisting of two aromatic rings attached by a methylene bridge, which is produced by 72 different plant species, including grapevines, pines, legumes, peanuts, soybeans and pomegranates [47]. The most common dietary source of resveratrol is red wine, and it is thought to be an influential factor in the French Paradox, a term describing the observation that the French population has a very low incidence of cardiovascular disease, in spite of a diet high in saturated fats [48].

This natural polyphenol seems to play an important role in neuroprotection in models of neurodegeneration (Alzheimer’s, Parkinson’s or Huntington’s disease), ischemia and brain and spinal cord injury [49,50]. The neuroprotective effects of RVT result from its antioxidant activity due to its stilbene structure with two phenol rings, that allows it to scavenge a variety of free radicals, including lipid peroxyl and carbon-centered radicals, reactive oxygen species, and because of its capacity to induce the expression of several antioxidant enzymes, such as SOD and GPx. Recent studies have revealed that resveratrol modulates the gene response related to redox pathways [51].

There are several evidences that demonstrate the efficacy of RVT in hypoxic-ischemic brain injury in the neonatal rat. Resveratrol significantly reduces brain injury, protecting against tissue loss measured at 7 days after the injury, by preserving neocortical and subcortical brain areas (sensorimotor cortex, hippocampus and striatum) and also neuronal networks responsible for learning and memory functions. In this way it ameliorates HI-induced short- and long-term behavioral deficits and significantly improves motor performance, and what is more, animals treated with resveratrol presented more cells morphologically well preserved than in the HI group [52-54]. It also maintains myelination, revealed by myelin basic protein (MBP) immunolabeling and Luxol Fast Blue (LFB), which means a significant reduction in white matter damage [52]. Resveratrol plays a neuroprotective role in HI brain damage through its anti-apoptotic effects, by means of decreasing the expression of Bax, Caspase-3 and the ratio of Bax/Bcl-2 and also
increasing Bcl-2 expression. It also reduces the calpain activation, suggesting that it works as a generally neuroprotective agent and not just on the apoptotic pathway [54-56]. Moreover, maternal dietary supplementation with pomegranate juice is neuroprotective for the neonatal brain, leading to markedly decreases in brain tissue loss (60%) in all three brain regions assessed and also to diminish caspase-3 activation by 84% in the hippocampus and 64% in the cortex [57,58].

Resveratrol has been found to be neuroprotective in the adult ischemia model in rats. Treatment with trans-RVT or RVT prevents motor impairment and neurological functions and significantly reduces the volume of infarct and brain edema in middle cerebral artery occlusion (MCAO) model of stroke in rats [58-60]. This natural polyphenol exerts its effect by improving brain energy metabolism (enhancing the level of glucose, ATP and energy charge, and decreasing the levels of lactate) and by attenuating oxidative stress. It inhibits xanthine oxidase activity and prevents the production of hypoxanthine, xanthine and oxygen radicals during ischemia/reperfusion [59]. In addition, it minimizes the generation of ROS, lipid peroxidation (MDA) and NO content and the expression of stress-associated proteins, including heat shock protein 70 (HSP70) and metallothionein [58,60,62]. RVT ameliorates the reduction in the total content of gangliosides, phospholipids, and cholesterol in hippocampus and cerebral cortex [61]. Administering resveratrol 7 days before global cerebral ischemia, resveratrol brings antioxidant and Nα(+)-K(+)-ATPase activity in cortex and hippocampus, return back to normal levels [62] and it remarkably reduces astrogial and microglial activation at 7 days after ischemia/reperfusion (I/R). It is thought that the neuroprotective effect of resveratrol preconditioning may be due in part to the suppression of the inflammatory response via regulation of NF-kB, COX-2 and iNOS induced by I/R [63]. Additionally, histological analysis of CA1 hippocampal region revealed that resveratrol treatment diminishes intercellular and pericellular edema and glial cell infiltration [60, 64], through activation of SIRT1, an NAD+-dependent deacetylase. Resveratrol increased expression of SIRT1 and phosphorylation of Akt and p38 but inhibited the increase in phosphorylation of ERK1/2 [64]. Resveratrol increases gene and protein levels of a downstream molecule of SIRT1 (peroxisome proliferator-activated receptor γ coactivator 1α), and mRNA levels of its target genes antioxidative SOD-2 and uncoupling protein 2. It also increases phosphorylation of cyclic AMP-response-element-binding protein and transcription of the anti-apoptotic gene Bcl-2 [62]. Moreover, its effects can be mediated through activation of the PI3-K-Akt signaling pathway and subsequently down-regulating expression of GSK-3β and CREB, thereby leading to prevention of neuronal death after brain ischemia in rats [65]. Taking together, these results suggest that the neuroprotective actions of this polyphenol, including anti-oxidative, anti-apoptotic and anti-inflammatory effects, are mediated via modulation of multiple signaling pathways in adult ischemia model in rats.

4.2 Docosahexaenoic acid

Docosahexaenoic acid (22:6n-3) is a long-chain omega-3 fatty acid, commonly found in fish such as salmon and tuna. It has 22 carbons with six double bonds (22:6), and the first double bond is three carbons from the methyl end of the molecule (hence omega-3). DHA is an essential dietary fatty acid because it, or its short chain precursor, alpha-linolenic acid (18:3 n-3), have to be obtained in the diet. In addition, dietary omega-3 fatty acid deficiency is associated with biochemical changes in the brain and with visual disorders [66-70]. In humans, DHA is present in lower concentrations in blood, but in very high concentrations in the brain, retina and spermatozoa. In fact, it is the major polyunsaturated fatty acid in the adult mammalian brain, where constitutes more than 30% of the total phospholipid composition of membrane, being present in three phospholipids (phosphatidilserine, phosphatidylethanolamine and ethanolamine plasmogen). DHA provides plasma membrane fluidity at synaptic regions so it is crucial for maintaining membrane integrity and, consequently, neuronal excitability and synaptic function. As a consequence, DHA is indispensable for maintaining membrane ionic permeability and the function of transmembrane receptors that support synaptic transmission and cognitive abilities [67, 71-73]. Inadequate dietary intakes of omega-3 fatty acids reduce DHA and augment omega-6 fatty acids in the brain.

Decreased DHA in the developing brain leads to deficits in neurogenesis, neurotransmitters metabolism, and altered learning and visual function in animals [74]. DHA accumulates in the brain during late prenatal and early postnatal development; in humans in the third trimester of pregnancy and in rats in the last 3 days of gestation [75]. Clinical trials have proved the importance of feeding term or premature infants with n-3 polyunsaturated acid, and of the maternal intake during pregnancy and lactation, since the omega-3 are provided during perinatal development through placental transfer and maternal milk, which determines the DHA status of the newborn and consequently impacts on post-natal development of brain and visual functions [74,76].

Docosahexaenoic acid significantly reduces brain volume loss and improves long-term neurological outcomes up to 5 weeks in a neonatal rat model of perinatal hypoxia-ischemia [77-78] and it exerts an anti-inflammatory effect in microglia by inhibiting NF-κB activation and subsequent release of inflammatory mediators [78]. Maternal DHA-enriched diet during pregnancy provides neuroprotection in neonatal brain injury by inhibiting oxidative stress (8-OHdG immunoreactivity were significantly decreased) and apoptotic neuronal death [75]. HI insults interfere with accumulation of brain DHA in developing rats, since 7 days after injury, the ratio of DHA to total fatty acids increases in the control group, but not in the HI group, suggesting that DHA supplementation may be beneficial for treating neonatal HI encephalopathy [78, 79]. In addition, DHA co-treatment with hypothermia produced both sustained functional improvement and reduced brain damage after neonatal hypoxia-ischemia [80].
In the adult model of stroke by MCAO, treatment with DHA significantly improves behavioral disturbance and reduces total infarct volume (by a mean of 40% when administered at 3h, by 66% at 4h and by 59% at 5h), edema and blood-brain barrier disruption [81,82], whereas chronic (but not acute) administration minimizes MDA levels and increases SOD activity after ischemic insult alleviating the oxidative stress in the rat brain [83]. DHA decreases necrosis, mainly modifying membrane biophysical properties and maintaining its integrity in functions between presynaptic and postsynaptic areas, triggering a better stabilization of the intracellular ion balance. DHA also palliates brain apoptosis as well, by inducing antiapoptotic effects; minimizing responses to ROS, upregulating anti-apoptotic and downregulating pro-apoptotic protein expression, and maintaining mitochondrial integrity and function. In animal models DHA renders neuroprotection after HI injury by regulating multiple molecular pathways and gene expression [84]. The protection of neuronal death is associated with increased Nrf2 activation and heme oxygenase-1 (HO-1) upregulation [85]. DHA ameliorates central macrophages/microglia activation, leukocyte infiltration and pro-inflammatory cytokine expression and peripheral leukocyte activation after cerebral ischemia [80].

Table 1  The mechanisms of action of RVT and DHA in the perinatal hypoxia-ischemia

<table>
<thead>
<tr>
<th>TARGET in PERINATAL HI</th>
<th>EFFECT of DHA (REFERENCES)</th>
<th>EFFECT of RESVERATROL (REFERENCES)</th>
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<tbody>
<tr>
<td>BRAIN PROTECTION</td>
<td></td>
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<tr>
<td>Infarct volume/Tissue loss</td>
<td>↓ [77,78]</td>
<td>↓ [52-54,56,58]</td>
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<tr>
<td>Apoptosis</td>
<td>↓ [75]</td>
<td>↓ [54-56]</td>
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<tr>
<td>Morphologically well preserved neurons</td>
<td>↑ [52-54]</td>
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<tr>
<td>White matter injury (MBP/LFB)</td>
<td>↓ [52]</td>
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<tr>
<td>Short-term behavioral deficits</td>
<td>↓ [52-54]</td>
<td></td>
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<tr>
<td>Long-term behavioral deficits (spatial learning and memory)</td>
<td>↓ [77,78,80]</td>
<td>↓ [52-54]</td>
</tr>
<tr>
<td>APOPTOSIS</td>
<td></td>
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<tr>
<td>Caspase-3</td>
<td>↓ [54-56,57]</td>
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<tr>
<td>Bax expression</td>
<td>↓ [54-56]</td>
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<tr>
<td>Bcl-2 expression</td>
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<td>Bax/ Bcl-2</td>
<td>↓ [54-55]</td>
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<td>Calpain</td>
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<tr>
<td>ANTIOXIDANT</td>
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<tr>
<td>DNA oxidation (8-OHdG)</td>
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<tr>
<td>INFLAMMATION</td>
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<tr>
<td>NF-κB</td>
<td>↓ [78]</td>
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5. Conclusion

Nowadays there are convincing evidences demonstrating that RVT and DHA treatments are effective against hypoxic-ischemic brain injury in the neonatal rat model, reducing infarct volume and neuronal loss, minimizing lipid and DNA peroxidation, blocking some apoptotic pathways, decreasing inflammation, inhibiting free radical production and increasing the production of some antioxidant enzymes such as GPx and SOD, showed in Figure 2 and Table 1. As hypoxic-ischemic injury is a complex process, an effective therapeutic effect will be obtained only by a strategy that can target multiple pathways. The use of synergic strategies, such as the association between DHA and resveratrol, might lead to a larger neuroprotective effect on the brain thus improving the neonatal outcome. However, there are not bibliographic evidences about the possible synergic pathways of RVT and DHA in hypoxia-ischemia, since it is known that RVT acts via SIRT1 pathways but the effectiveness and precise modes of the neuroprotective action of DHA remain incompletely understood.

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