



## Maternal alcoholism and neonatal hypoxia-ischemia: Neuroprotection by stilbenoid polyphenols

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**Title: Maternal alcoholism and neonatal hypoxia-ischemia: neuroprotection by stilbenoid polyphenols**

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## **Abstract**

The impact of maternal nutrition on neurodevelopment and neonatal neuroprotection is a research topic with increasing interest. Maternal diet can also have deleterious effects on fetal brain development. Fetal exposure to alcohol is responsible for poor neonatal global development, and may increase brain vulnerability to hypoxic-ischemic (HI) encephalopathy, one of the major causes of acute mortality and chronic neurological disability in newborns. Despite frequent prevention campaigns, about a quarter of women in the general population drinks alcohol during pregnancy and breastfeeding. This study was inspired by this alarming fact. Its aim was to evaluate the beneficial effects of maternal supplementation with two polyphenols during pregnancy and breastfeeding, on hypoxic-ischemic (HI) neonate rat brain damages, sensorimotor and cognitive impairments, in a context of moderate maternal alcoholism. Both stilbenoid polyphenols, *trans*-resveratrol (RSV - 0.15 mg/kg/day), and its hydroxylated analog, *trans*-piceatannol (PIC - 0.15 mg/kg/day), were administered in the drinking water, containing or not alcohol (0.5 g/kg/day). In a 7-day post-natal rat model of HI, our data showed that moderate maternal alcoholism does not increase brain lesion sizes measured by MRI but leads to higher motor impairments. RSV supplementation could not reverse the deleterious effects of HI coupled with maternal alcoholism. However, PIC supplementation led to a recovery of all sensorimotor and cognitive functions. This neuroprotection was obtained with a dose of PIC corresponding to the consumption of a single passion fruit per day for a pregnant woman.

**Highlights:**

- Maternal alcoholism increases deficits induced by neonatal hypoxia-ischemia (HI).
- These impairments are counteracted by maternal supplementation with piceatannol.
- Doses used are equivalent to the piceatannol content of a single passion fruit.
- Piceatannol is neuroprotective in neonatal HI in a context of maternal alcoholism.
- Originality of the study: nutritional and transgenerational therapeutic approach.

**Key words:** neonatal hypoxia-ischemia, neuroprotection, polyphenols, magnetic resonance imaging, maternal supplementation, maternal alcoholism

## 1. Introduction

Neonatal hypoxia-ischemia (HI) is an important socio-sanitary problem as a major cause of morbidity and mortality in human newborns (1-6/1,000 births) with a high risk of chronic motor, behavioral and neurological deficits <sup>1, 2</sup>. HI is characterized by a cerebral deprivation of oxygen and glucose leading to cell death, the most affected being neurons <sup>3, 4</sup>, which leads to sensorimotor deficits and mental retardation associated to learning abnormalities <sup>5, 6</sup>. Despite this major public health problem, the only current clinical treatment is hypothermia, which is thought to slow down neuronal cell death processes by reducing brain metabolism <sup>7-9</sup>. This unique therapeutic solution does not appear sufficiently successful (44–53% of infants die or suffer moderate to severe neurological disability after hypothermia treatment <sup>10</sup>) and new therapeutic approaches must be developed to possibly reverse neonatal HI sequelae. In parallel to this neonatal public health problem, despite better communication about the risks, alcohol consumption in Western countries of pregnant women has not decreased <sup>11</sup>. In the USA, 1 out of 12 pregnant women drink alcohol <sup>12</sup>. Maternal alcohol consumption during pregnancy and breastfeeding is well known to lead to deleterious effects on neurodevelopment. It represents the major cause of intellectual disability in neonates <sup>13</sup>, a phenomenon called fetal alcohol syndrome, which incidence is 1/500 births <sup>14</sup>. In HI context, it has been shown *in vitro*, on primary neuronal cultures, that combination of ethanol, even at moderate doses, and hypoxia (0.01% ethanol during 24 h plus 0.1% O<sub>2</sub> during 30 min) promote synergistically neuronal death <sup>15</sup>. Therefore, neonatal HI in a maternal alcohol exposure context needs better investigations.

We used the classical Rice-Vannucci rat HI model <sup>16</sup>, which briefly consists of a permanent unilateral occlusion of the common carotid artery, followed by a hypoxic episode, in 7-days old pups, an age that corresponds to a full-term newborn. This HI model induces large ipsilateral striatal, hippocampal and cortical injuries <sup>17</sup>, leading to sensorimotor and cognitive deficits <sup>18</sup>.

The impact of maternal nutrition on neonatal neurodevelopment and on neonatal neuroprotection is a research topic with increasing interest <sup>19, 20</sup>. Maternal dietary supplementation with ingestion of pomegranate juice (32, 16 or 8  $\mu$ mol of polyphenols/day), which contains important quantities of polyphenols, was shown to lead to significant neuroprotection <sup>20</sup>. In another study, in a dam supplemented with a pomegranate polyphenol extract (4.8 mg polyphenols per day, orally administered during pregnancy and after delivery), a neuroprotective effect on neonatal brain injury after HI was also observed <sup>21</sup>. These authors also tested direct intraperitoneal *trans*-resveratrol (RSV) administration on pups. RSV injection (200  $\mu$ g/kg or greater) reduced caspase-3 and calpain activation and could protect neonatal brain against HI injuries. RSV, notably found in grape skins and several types of nuts or roots is one of the most extensively studied polyphenols. It can cross the placenta <sup>22</sup> and its maternal consumption during pregnancy is safe <sup>23</sup>. Although the therapeutic properties of RSV are well documented, its bioavailability is low (<1%). Its hydroxylated analogue, *trans*-piceatannol (PIC), found in large quantities in the seeds of passion fruit, has a two-fold higher bioavailability <sup>24</sup>. However, very few studies have been carried out with this compound and only one study has been conducted on neonatal HI <sup>25</sup>. In this study, we show that PIC maternal supplementation (0.15 mg/kg/d) is neuroprotective by decreasing the incidence of HI lesion, decreasing the lesion volume and counteracting the deleterious effects on sensorimotor and cognitive abilities of pups.

The aim of the present work was to study the effect of a maternal supplementation with RSV or PIC on HI-pup brain damage in a context of maternal alcoholism. Pregnant and nursing dams were supplemented with RSV or PIC and/or ethanol and the Rice-Vannucci rat model was used to induce HI brain injury in their post-natal seven-day-old pups (P7). We evaluated *in vivo* neonate brain damages and their evolution with noninvasive magnetic resonance imaging (MRI). Cell death and brain edema were quantified in young rats for each maternal nutritional condition. Finally, motor

and behavioral studies were carried out to evaluate the effect of polyphenols on cognitive function altered by HI event and maternal alcoholism.



## 2. Results

### *2.1. Piceatannol but not resveratrol reduces neonatal brain injuries after an HI episode, in a moderate maternal alcoholism context*

Typical apparent diffusion coefficient (ADC) maps of Diffusion Weighted Image (DWI) of P7-pup brains (3 h post-ligation) are presented in Fig. 1A. As expected, the sham group did not show any lesions. In all other groups, HI induces brain damages (Fig. 1A, hyposignal). HI injury affected more particularly the cortex, the hippocampus and the striatum. At P7, no difference in lesion volumes was detected between the different HI groups (Fig. 1B). At P9, pups that underwent an HI episode and whose dam was supplemented with both PIC and a moderate dose of ethanol (PIC/Eth group) had significantly lower lesion volumes than control HI pups (HIC group; pups with HI and coming from dam without any supplementation) and HI pups from dam supplemented with ethanol (Eth group) ( $8\% \pm 1\%$  for PIC group vs  $19\% \pm 4\%$  and  $18\% \pm 2\%$  for HIC and Eth groups, respectively).

DWI allows the basic measure of ADC. This value corresponds to the displacement rate of extracellular water molecules; the smaller, the more severe the cytotoxic edema. Typically, ADC values decrease within minutes of brain injury as a reflection of this cytotoxic edema. The change in ADC value after HI is a dynamic process and tends to return to higher values 24 to 48 h after the insult, a phenomenon called “pseudonormalization”<sup>26</sup>. ADC values at P7 were quantified in cortex, hippocampus and striatum (Fig. 1C). ADC significantly decreased in all HI groups. The severity of edema in the cortex and the hippocampus was significantly lower in the Eth and PIC/Eth groups compared to the other HI groups.

Neuronal death was evaluated in the penumbra region (cortex and striatum) by Nissl staining on P9-pups (Fig. 1D). For the cortical region, the HIC group presented the highest level of neuronal death compared to other HI groups (Fig. 1E). No significant difference was measured between Eth

and RSV/Eth groups. However, less neuronal death was measured in the PIC/Eth group compared to the RSV/Eth group ( $7\% \pm 6\%$  vs  $21\% \pm 4\%$ , respectively). In the striatal region, the PIC/Eth group presented the lowest level of neuronal death compared to other HI groups and both Eth and RSV/Eth groups presented the highest level of neuronal death.

## *2.2. Maternal polyphenol supplementation allows recovery of neonatal HI brain lesions detected by MRI despite moderate maternal alcoholism*

At P30, 23 days after the HI insult, brain lesions were still observed by MRI in all pups in the HIC and Eth groups, whereas no more lesion was detected for 20% and 50% of pups in the RSV/Eth and the PIC/Eth groups, respectively (Fig. 2A). Pups in the HIC, Eth and RSV/Eth groups had larger lesion volumes compared to pups in the PIC/Eth group (Fig. 2B). Even if the incidence of brain damage was decreased by maternal RSV supplementation, RSV administration did not reduce lesion volumes (Fig. 2B,  $31\% \pm 5\%$ ;  $27\% \pm 3\%$ ;  $24\% \pm 7\%$ ;  $12\% \pm 6\%$ , in HIC, Eth, RSV/Eth and PIC/Eth groups, respectively).

## *2.3. Piceatannol counteracts the deleterious sensorimotor and cognitive effects induced by neonatal HI, despite moderate maternal consumption of ethanol*

Cortex and striatum, two structures involved in automatic motricity, were almost systematically damaged in our HI model.

The righting test was performed at P8, P10 and P12 to evaluate the impact of the different maternal supplementations on sensorimotor abilities and neurological reflexes after HI (Fig. 3A). Pup performances improved with time, reflecting a process of learning and habituation, in all groups except in the Eth group. Best performances were measured for sham pups and pups from the PIC/Eth group (no significant difference between these two groups). A delay in learning was

observed for pups in the HIC and RSV/Eth groups compared to the ones in sham and PIC/Eth groups. Pups in the HIC and RSV/Eth groups showed intermediate performances between sham or PIC/Eth groups and Eth group, in which pups had the poorest performances.

The same pattern was observed in the negative geotaxis test. Pups from the Eth group showed the worst performances compared to the other HI groups and pups from the PIC/Eth group had identical abilities than the sham group (no significant difference between sham and PIC/Eth, whatever the considered test day) (Fig. 3B).

Since the HI model (unilateral carotid ligation) induces ipsilateral lesions, sensorimotor asymmetries were assessed using the adhesive removal test (Fig. 3C). This test allowed to discriminate between pups from the Eth group and all other pups since they had the poorest performances. The time to remove the adhesive was decreased with days, indicating that animals were learning the task, for all pups except the ones in the Eth group. Pups in the RSV/Eth group presented intermediate abilities while HIC and PIC/Eth groups performed as well as the sham group (no significant difference between sham, HIC and PIC/Eth groups).

Sensorimotor deficits were also assessed using the modified neurological severity score (mNSS) (Fig. 3D). Animals were evaluated at P24 (17 days post-HI). The mNSS score in sham and PIC/Eth groups was significantly lower than the one measured in HIC, Eth and RSV/Eth groups ( $0.38 \pm 0.18$ ;  $0.83 \pm 0.31$ ;  $4.86 \pm 0.88$ ;  $5.0 \pm 0.47$  and  $4.83 \pm 1.24$  for sham, PIC/Eth, HIC, Eth and RSV/Eth groups, respectively).

In our HI model, hippocampi were frequently injured. For this reason, the novel object recognition test was used to evaluate hippocampal-dependent long-term memory of pups at a later stage (Fig. 3E). Animals were tested at P45. Juvenile rats in the HIC and Eth groups presented a long-term memory deficit, whereas no difference was found between animals in the sham and PIC/Eth groups. Rats in the RSV/Eth group presented an intermediate ability to discriminate the novel object

(discrimination index:  $0.77 \pm 0.04$ ;  $0.47 \pm 0.09$ ;  $0.41 \pm 0.04$ ;  $0.58 \pm 0.09$ ;  $0.66 \pm 0.02$  for sham, HIC, Eth, RSV/Eth and PIC/Eth, respectively).

### 3. Discussion

Neonatal HI caused by oxygen and glucose deprivation induces an energetic failure and a decrease in ATP, which in turn leads to a reduced sodium-potassium ATP pump activity and membrane depolarisation. The loss of membrane polarity leads to glutamate efflux and calcium influx <sup>27, 28</sup>. Calcium is responsible for the activation of several enzymes such as proteases, endonucleases and nitric oxide synthase <sup>29</sup>. Therefore, the initial oxygen and glucose deficits lead to excitotoxicity, disruption of ion homeostasis and calcium dysregulation that affect mitochondria. In a second step, during reperfusion, due to this first energetic failure and the increase in free radicals and reactive oxygen species (ROS) production, cellular swelling takes place and neuronal apoptosis occurs. Indeed, neurons are the most affected by oxygen and glucose deprivation <sup>3, 4</sup>. Their death results in sensorimotor deficit, mental retardation and learning abnormalities <sup>5, 6</sup>. The modest response to the unique therapeutic strategy proposed up to now (hypothermia) emphasizes the necessity to find new treatments.

A growing number of studies have shown a neuroprotective role of RSV in a context of neonatal HI. This polyphenol was found to be a potent protective agent after an HI event by reducing infarct size, neuronal loss and, consequently, by preventing cognitive deficits <sup>21, 30, 31</sup>. However, the neuroprotective effect of RSV was observed after direct intraperitoneal injection in pups that underwent HI, a pro-inflammatory mode of administration. Moreover, in previous studies, RSV was administrated at high pharmacological doses (20-100 mg/kg). In this study, we used, for the first time, a low (nutritional) dose of RSV (0.15 mg/kg/day) and explored the impact of maternal supplementation. This corresponds to an ingested doses of 10.5 mg of RSV or PIC per day for a pregnant woman (weighing 70 kg). RSV is present in various edible plants such as rhubarb, fresh peanut, cocoa beans and in large quantities in grapes, which contain up to 486 mg/kg of RSV<sup>32</sup> (depending on the specie). Therefore, the doses of RSV administered during our maternal

supplementation protocol correspond to the consumption of about 22 g of grapes (about thirty grape berries) per day for a pregnant woman. Similarly, taking into account the PIC content of passion fruit (2.2 mg of PIC per g of raw passion fruit seeds<sup>33</sup>) and the weight of the seeds (between 5 and 10 g/fruit), a passion fruit contains about 10 to 20 mg of PIC. Therefore, the doses of PIC tested in maternal supplementation in the rat correspond to the consumption of a single passion fruit per day for a pregnant woman. Concerning ethanol, the doses used to mimic maternal alcoholism are moderate. Since dams were supplemented with ethanol at a dose of 0.5 g/kg/d, this corresponds to 35 g/d for a pregnant woman (70 kg), which is equivalent to the consumption of 2 glasses of wine (14 cl, 12.5 °). This dose was chosen to correspond to a moderate alcohol consumption and our model reflects the fact that 10% of women still drink alcohol during pregnancy<sup>34</sup>. If the prevalence of alcohol use during pregnancy was estimated by several studies, it is more difficult to get epidemiological data on doses of alcohol intake during this period. However, it has been estimated that among pregnant women drinking a glass of wine daily, 10% will have a child with fetal alcohol syndrome<sup>35</sup>. Therefore, the main aim of the present original nutritional and transgenerational study was to evaluate the effect of a maternal supplementation with RSV or PIC on HI-pup brain damages in a context of moderate maternal alcoholism.

Besides the quantification of lesion volume, the early measurements of ADC reflected cytotoxic edema severity. Indeed, in this case, tissue edema is due to cellular swelling and leads to a decrease in extracellular water movement speed and then, therefore, to a decrease in ADC values. Three major types of cerebral edema have been described: cytotoxic, ionic and vasogenic<sup>36</sup>. Cytotoxic edema occurs in the early stage after stroke and is characterized by the swelling of astrocytes and neuronal dendrites<sup>37, 38</sup>, due to the energetic failure linked to oxygen and glucose deprivation, which leads to a cellular entry of ions and water. Ionic edema has been described as a continuum between cytotoxic and vasogenic edema and is characterized by the swelling of endothelial cells.

It occurs within 30 minutes after reperfusion<sup>36</sup>. The vasogenic edema is linked to the blood-brain barrier disruption and happens at a later stage<sup>37</sup>. A smaller decrease in ADC values in the cortex and in the striatum was measured in the PIC/Eth group indicating that, in addition to have smaller lesions, pups that underwent HI in this group presented a less severe cytotoxic edema. These data underlined a neuroprotective role of maternal PIC supplementation. This neuroprotective effect of PIC supplementation was also observed at the cellular level by histochemistry since less neuronal death was measured in both cortical and striatal penumbra regions. Surprisingly, maternal Eth consumption also led to a smaller decrease in ADC values but was, however, neither linked to smaller lesion volumes 48 h post-HI nor with neuronal survival determined by the Nissl staining. This underline the fact that ADC values alone, measured just after the insult, are not sufficient to predict the recovery of the penumbra zone.

Regarding sensorimotor performances, hypoxic-ischemic pups (HIC) performed less well than pups in the sham group for the righting reflex and the negative geotaxis tests. These results are in agreement with previous studies on mice or rats subjected to HI<sup>39-41</sup> showing that this event affects the short-term outcome of early reflexes. The deleterious effect of maternal alcoholism could be highlighted by these tests since Eth group pups were not able to improve their performance with time, which was the case for pups in the other HI groups. If maternal supplementation with RSV allowed to partially counteract some deleterious effects of HI, this supplementation was not as neuroprotective as PIC. Indeed, pups in the PIC/Eth group were able to reach the same level of performance as sham pups for these tests, meaning that maternal oral PIC supply improves early deficits after HI injury, even in a context of maternal alcoholism. Pups subjected to neonatal HI show a high capacity of recovery, as it has been suggested by previous studies<sup>39,41</sup> and RSV has been shown to decrease the deleterious effects of Eth in hippocampal neurogenesis, in 7-day-old mice<sup>42</sup>. Sensorimotor functions were also assessed later in juvenile rats (P20-24) using the adhesive

removal test. This test is usually used to detect long-term deficit after stroke in adult rodents <sup>43-45</sup> and was adapted to juvenile rats by Bouet *et al.* for assessing somatosensory deficits and asymmetric dysfunctions after neonatal ischemic stroke <sup>43</sup>. In this test, even if all groups, except the Eth group, improved their performance over time, as a consequence of task learning, HI groups still performed less well than the sham group except the PIC/Eth group which was indistinguishable from the sham group. These results indicate that maternal PIC supplementation counteracted the deleterious effect of the HI event on juvenile somatosensory asymmetries even in a maternal alcoholism context. These data were reinforced by the mNSS results.

It had been reported in a cohort of 40 children that HI can lead to hippocampal damage and, later to an impairment in cognitive memory<sup>46</sup>. HI rodents also display short- and long-term memory deficits <sup>31, 47-49</sup>. Long-term memory was assessed by the novel object recognition test, in which maternal PIC supplementation was able to revert the deleterious effect of HI and maternal alcoholism (same discrimination index for PIC/Eth and sham groups). These data show a neuroprotective role of maternal PIC supplementation on hippocampal-dependent long-term memory of pups. These results are in agreement with the study of Arteaga *et al.* <sup>31</sup>, which demonstrated that intraperitoneal injection of RSV improves the long-lasting cognitive deficits induced by HI (a study not in the context of alcoholism however). They proposed that the RSV protective effect could be due to the protection and preservation of neocortical and subcortical brain areas (sensorimotor cortex, hippocampus and striatum) and in particular, of neuronal networks responsible for learning and memory <sup>31</sup>. The authors postulated in this article that the neuroprotective role of RSV could be due to its antioxidant effect. Pups in the Eth group presented the lowest discrimination index, indicating that long-term memory was impaired, even in a context of moderate maternal alcohol consumption. Such an impairment was also observed in a context of mild focal traumatic brain injury coupled with alcohol exposition (sub-chronic intermittent alcohol



vapour (95% ethanol 14h on/10h off) <sup>50</sup>. It could be noted also that pups in the RSV/Eth group tended to present intermediate discrimination index values, suggesting that RSV could prevent, but only partially, hippocampal-dependant long-term memory deficits induced by ethanol consumption.

Many studies have focused on the antioxidant properties of RSV. However, these properties are observed only at high doses. In the context of HI, RSV neuroprotection could be multimodal. In addition to SIRT1 activation <sup>51</sup>, RSV could also modulate metabolism (Fig. 4). For example, HI leads to a decrease in astrocytic glutamate uptake <sup>52</sup>, which results in glutamate excitotoxicity that could be reversed by RSV i.p. injection in a rat model of transient forebrain ischemia <sup>53</sup>. The same was observed in a rat hippocampal slice model of H<sub>2</sub>O<sub>2</sub>-induced oxidative injury <sup>54</sup>. In another study, in rat hippocampal slices exposed to glutamate, the astrocytic recycling of glutamate to glutamine was increased after RSV exposure (100  $\mu$ M). Similar data were also obtained in primary astrocyte cultures of P1-pups in presence of RSV (10  $\mu$ M) <sup>55</sup>. Moreover, in primary cultures of mouse cortical astrocytes, it has been shown that the uptake of glutamate induces an increase in astrocytic production of lactate *via* the stimulation of glycolysis <sup>56</sup>. Interestingly, RSV has been shown to increase glycolysis in the liver <sup>57</sup>, but also in the brain, leading to a neuroprotective effect in a mouse model of middle cerebral artery occlusion <sup>58</sup>. In parallel, in rat hippocampal slices, RSV (100  $\mu$ M) led to an increase in lactate release <sup>59</sup>. Although lactate has long been considered as a metabolic waste product, it is now more and more considered as a substantial brain energetic fuel. In 1994, Pellerin and Magistretti proposed a metabolic exchange between astrocytes and neurons in which blood glucose enters first the astrocytes, which are more glycolytic cells <sup>60</sup>, where it is converted into lactate. This lactate is then transferred to neurons where it is converted back to pyruvate for its oxidative use<sup>61</sup>, an intercellular exchange called the astrocyte-neuron lactate shuttle (ANLS) <sup>56</sup>. In the context of HI, providing lactate as an energetic substrate for neurons could spare

glucose for its metabolic use through the pentose phosphate pathway (PPP). Indeed, this pathway is mandatory to maintain high levels of reduced glutathione, a metabolite necessary to fight against ROS. Glutathione levels were measured in neuronal stem cells treated with RSV (5  $\mu$ M) following oxygen-glucose deprivation/reoxygenation<sup>62</sup> as well as in rat hippocampal slices treated with RSV (100  $\mu$ M), in a context of H<sub>2</sub>O<sub>2</sub> oxidative stress injury<sup>54</sup>. The content of glutathione was increased by 20% and 80%, respectively. In astrocytes, RSV (10 mM) was able to decrease intracellular ROS levels induced by H<sub>2</sub>O<sub>2</sub> oxidative stress<sup>63</sup>. By increasing glycolysis, and therefore lactate, in astrocytes (which could then promote glucose use *via* PPP in neurons and enhanced their reduced glutathione levels), RSV may prevent neuronal death and reduce brain damages leading to motor and cognitive impairments.

In this study, RSV was not able to fully protect against HI lesions in a context of moderate alcohol maternal intake. However, PIC supplementation, even combined with a moderate dose of ethanol, allowed pups to recover their sensorimotor and cognitive functions after an HI event. PIC differs from its analogue RSV by only one hydroxyl group. This confers to PIC a higher bioavailability compared to RSV<sup>24</sup>, which could explain the higher neuroprotective effect of PIC compared to RSV. Contrary to a comprehensive literature on RSV, few publications report on the effects of PIC; our results argue in favor of the need to continue studies on this molecule.

Taken altogether, our results support that in a context of neonatal HI and moderate maternal alcohol consumption, PIC, at a dose equivalent in humans to 1 passion fruit / day, could partially reverse the anatomical brain lesions and, more importantly, could totally counteract the sensorimotor and long-term memory impairments. Based on these results, maternal supplementation with PIC may be considered as a preventive nutritional approach in the context of maternal alcoholism to limit further possible brain damages if a neonatal HI event might occur.

## 4. Experimental procedures

### 4.1. Study design

The aim of the present work was to study the effect of maternal nutritional supplementation with polyphenols on offspring brain damages induced by HI, in a moderate maternal alcoholism context. Scheme of the experimental design is presented in Fig. 5. All animal procedures were conducted in accordance with the Animal Experimentation Guidelines of the European Communities Council Directive of November 24, 1986 (86/609/EEC). Protocols met the ethical guidelines of the French Ministry of Agriculture and Forests, and were approved by the ethical committees (N° authorization 9476). Sample size was determined based on a previous study <sup>64</sup>.

### 4.2. Animals

Pregnant Wistar RJ-HAN females (Janvier Laboratories, Le Genest Saint Isle, France) were received one week before delivery and were kept on a 12:12 h light:dark cycle with water and food (A03) *ad libitum*.

### 4.3. Model of hypoxic-ischemic brain injury

Cerebral HI was performed as described previously <sup>16,25,65</sup>. Briefly, P7-pups of either sex, weighing 14-20 g, were anaesthetized with isoflurane (induction: 4%; maintenance: 1.5%) and a midline neck incision was made under local anesthesia (lidocaine 0.5%). The left common carotid artery was exposed and permanently ligated (7-0 silk thread). After surgery, animals were allowed to recover (30 min) on a heated mattress to maintain body temperature at  $36 \pm 1^\circ\text{C}$ . Pups were then placed for 2 h in a humidified hypoxic chamber (8% oxygen, 92% nitrogen –  $33^\circ\text{C}$ ) (Intensive Care Unit Warmer, Harvard Apparatus, Les Ulis, France) to maintain a constant thermal environment (rectal temperature:  $36 \pm 1^\circ\text{C}$ ). During hypoxia, sham-operated pups were kept separated from the

dam in a heated atmosphere (33°C). The first diffusion MRI was performed 3 h after carotid ligation, then after 48 h (P9) and 23 days (P30).

#### *4.4. Experimental groups*

Five experimental groups were considered. The different nutritional supplementations were added in the maternal drinking water 7<sup>th</sup> day before delivery until the 9<sup>th</sup> day post-delivery.

Maternal drinking water was supplemented with Eth (Sigma-Aldrich, France; 0.5 g/kg/d - equivalent to the consumption of 2 glasses of wine (14 cl, 12.5 °) for a pregnant woman) for Eth group (n=19), Eth (0.5 g/kg/d) and RSV (Sigma-Aldrich, France; 0.15 mg/kg/d) for RSV/Eth group (n = 10) and Eth (0.5 g/kg/d) and PIC (Tokyo Chemical Industry Co, France; 0.15 mg/kg/d) for PIC/Eth group (n = 12). For sham (n = 8) and control groups (HIC group, n = 17), 4 µl of absolute Eth were added into the rat water bottle (corresponding to the Eth volume added during polyphenols supplementation (final Eth concentration: 0.002%) since polyphenol solutions were prepared in absolute ethanol).

Additional controls were also performed to ensure that maternal RSV or PIC supplementation alone was not deleterious for pups (MRI and behavioural parameters, data not shown). For these two supplementary groups, dams were supplemented with RSV (n = 8) or PIC (n = 8) (0.15 mg/kg/d), respectively, during the same period.

#### *4.5. Acquisition of in vivo longitudinal MRI.*

*In vivo* MRI was performed at P7 (3 h after carotid ligation), P9 and P30 on a horizontal 4.7T Biospec 47/50 system (Bruker, Ettlingen, Germany) equipped with a 6-cm BG6 gradient system (1000 mT/m,  $\Delta=20\text{ms}$ ,  $\delta=4\text{ms}$ ). For MRI, pups were anesthetized with isoflurane 4% for induction and  $1.5 \pm 1\%$  for maintenance. Respiration was monitored by a ventral pressure sensor. Pup body

temperature was maintained at  $36 \pm 1^{\circ}\text{C}$  during acquisitions with a water-heated MRI bed. Anatomical T2-weighted images of the brain were obtained using a Rapid Acquisition with Relaxation Enhancement (RARE) sequence with 20 axial slices, 0.7 mm thick, echo time (TE) = 50 ms, repetition time (TR) = 3000 ms (total duration 4 min 48 s). Brain lesions were assessed by Diffusion Weighted Imaging (DWI: 20 axial slices of 0.7 mm, 30 directions, b-value  $1347 \text{ s/mm}^2$ , TE 24 ms, TR 2 s, total duration 17 min 04 s). Measurements were performed with Paravision 6.0.1 software (Bruker BioSpin, Karlsruhe, Germany).

#### *4.6. MRI Analysis*

The lesion volumes were measured on the ADC maps on 20 successive slices at P7, P9 and on T2 weighted images at P30 and are presented as a % of the total brain volume. For each rat, two region-of-interest (ROI) were manually drawn, on each slice ( $n = 20$ ), the first one that encompasses the injured area and the second one to determine the global brain area. Edema severity was evaluated by the measurement of ADC values at P7. ADC maps were computed using the Paravision 6.0.1 software diffusion tensor calculation module. ADC values were measured inside the lesion area. ROIs were manually delineated in each injured structure (cortex, hippocampus, striatum). Analyses were performed blind by 2 separate operators (intra-operator variability  $< 1\%$ ; inter-operator variability  $< 5\%$ ).

#### *4.7. Behavioral study*

##### *4.7.1. Righting reflex (P8, P10, P12)*

Pups were placed on their back and the time they took to turn over their four paws was recorded. Each pup was given three trials a day and the mean time to perform the test was calculated.

#### *4.7.2. Adhesive removal test (from P20 to P24)*

Somatosensory deficits and asymmetric dysfunctions were evaluated using the adhesive removal test<sup>43, 66</sup>. After an habituation period (60 s) in the open field (20 cm x 30 cm), two adhesive tapes (0.5 cm x 0.5 cm) were used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. Time to contact (control, no significant difference between groups, data not shown) and to remove the adhesive were measured during one trial per day (120 s maximum).

#### *4.7.3. Modified neurological severity score (mNSS) (P24)*

Neurological functions were assessed using the mNSS<sup>66</sup>, which is composed of motor (muscle status), sensory (tactile and proprioceptive), beam balance and reflexes (pinna, corneal and startle) tests. Each rat was graded on the mNSS scale (<1: no impairment – 1-6: middle impairment – 7-12: moderate impairment – 13-18: severe impairment).

#### *4.7.4 Novel object recognition (P45)<sup>31</sup>*

Hippocampal-dependent long-term memory of pups was assessed using the novel object recognition test, as described previously<sup>25</sup>. Briefly, after an habituation period (10 min) in the open field (50 cm x 50 cm) on day 1, pups were further allowed to freely explore two identical objects placed in opposite corners of the arena for 10 min during the following two days. On the day after (day 4), one of the previous object was replaced by a different one and pups were allowed to freely explore the objects for 10 min. Duration of object exploration was measured. The discrimination index was calculated as followed:  $([\text{time spent on new object} - \text{time spent on familiar object}] / [\text{time spent on new object} + \text{time spent on familiar object}])$ .

#### *4.7.5 Nissl staining*

In addition to MRI and behavioral tests, Nissl staining was performed on P9-pups brain sections, as described previously<sup>25</sup>, to highlight neuronal death in the penumbra region. Briefly, pups were anesthetized (isoflurane 4.5%) and were perfused transcardiacally first with PBS (1X) then with paraformaldehyde (PFA 4%) for 10 min. Removed brain were post-fixed in PFA over night, cryoprotected by sucrose immersion and then frozen in liquid N<sub>2</sub> vapor. The 16µm-thick brain sections were stained with 0.5% cresyl violet (Sigma Aldrich, France) for 5 min and washed in distilled water for 10 s.

#### *4.8. Statistical Analysis*

Statistical analysis and graphs were performed using GraphPad Prism 7.00 software. All data were expressed as means  $\pm$  standard error of the means (SEM). Statistical significance of the differences between multiple groups was determined using One-Way ANOVA followed by a Fisher's LSD post hoc test. Statistical significance was defined as  $p < 0.05$ . Number of pups was stated with each result along with the statistical test used.

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#### *4.10. Conflict of Interest*

Authors declare that they have no conflict of interest.

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## Figure legends

### **Fig. 1 Early impact of maternal nutritional supplementation on neonate brain injuries**

**induced by HI.** (A) Typical DWI of P7-pup brains of sham, HIC, Eth, RSV/Eth, and PIC/Eth groups. Images were obtained at 4.7 T, 3 h after injury. Damages appeared as a hyposignal. (B) Quantification of lesion volumes for the different groups between P7 and P9 (% , normalized to total brain). (C) Ipsilateral ADC values for sham, HIC, Eth, RSV/Eth and PIC/Eth groups. (D) Nissl staining in the penumbra region from P9-pups for the different groups. (E) Neuronal death evaluated in the penumbra region after Nissl staining. Results are mean values  $\pm$  SEM. \*Significant difference between two groups (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*\*  $p < 0.0001$ ), one-way analysis of variance (ANOVA) with Fisher's LSD post hoc tests;  $n = 8$  sham pups,  $n = 17$  Ct pups,  $n = 19$  Eth pups,  $n = 10$  RSV/Eth pups,  $n = 7$  PIC/Eth pups.

### **Fig. 2 Long term impact of maternal nutritional supplementation on neonate brain injuries**

**induced by HI.** (A) Representation of the percentage of pups still presenting brain lesions for HIC, Eth, RSV/Eth and PIC/Eth groups, at P30. (B) Lesion volume for HIC, Eth, RSV/Eth and PIC/Eth groups, at P30. Results are mean values  $\pm$  SEM. \* Significant difference between two groups (\*  $p < 0.05$ ), one-way analysis of variance (ANOVA) with Fisher's LSD post hoc tests;  $n = 6$  HIC pups,  $n = 8$  Eth pups,  $n = 5$  RSV/Eth pups,  $n = 6$  PIC/Eth pups.

### **Fig. 3 Early and long-term effect maternal supplementation on sensorimotor and cognitive**

**functions of HI pups.** (A) Performance for righting reflex for sham, HIC, Eth, RSV/Eth and PIC/Eth groups, from P8 to P12. (B) Performance for negative geotaxis for sham, HIC, Eth, RSV/Eth and PIC/Eth groups, from P8 to P12. (C) Performance for adhesive removal test between P19 and P23, for sham, HIC, Eth, RSV/Eth and PIC/Eth. (D) mNSS scores at P24 for each group. (E) Performances for the novel object recognition test, assessed at P45 for sham, HIC, Eth, RSV/Eth and PIC/Eth groups. The discrimination index was calculated as: [time in new object –

*time in familiar object]/[time in new object + time in familiar object]. Results are mean values  $\pm$  SEM. \* significant difference between two groups ( \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ), one-way analysis of variance (ANOVA) with Fisher's LSD post hoc tests;  $n \geq 8$  sham pups,  $n \geq 7$  HIC pups,  $n \geq 9$  Eth pups,  $n \geq 5$  RSV/Eth pus,  $n \geq 6$  PIC/Eth pups.*

**Fig. 4 Schematic representation of RSV effects on brain metabolism.**

**Fig. 5 Scheme of experimental design.** *Five different groups were included in the study: sham group that only suffered the exposition of the left common carotid artery (no HI), HIC group that was exposed to HI procedure, Eth group whose dams were supplemented with Eth (0.5 g/kg/d), RSV/Eth group whose dams were supplemented with RSV (0.15 mg/kg/d) plus Eth (0.5 g/kg/d), and PIC/Eth group whose dams were supplemented with PIC (0.15 mg/kg/d) plus Eth (0.5 g/kg/d). HI brain lesions were followed-up by MRI. Effects of maternal supplementation on HI brain damages were assessed by histological analysis as well as a sensorimotor and behavioral approach.*