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Fetal asphyxia induces a preconditioning effect in the cerebral prefrontal cortex

Diego Alexander Cruz Pico

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Fetal asphyxia induces a preconditioning effect

in the cerebral prefrontal cortex

Diego Alexander Cruz Pico

Marco Fornasini, MD, Ph.D
Director de Tesis y
Miembro del Comité de Tesis

Manuel Baldeon, MD, Ph.D
Miembro del Comité de Tesis

Winfried Wojtenek, Ph.D
Miembro del Comité de Tesis

Enrique Noboa, MD
Decano del Colegio de Ciencias de la Salud

Quito, abril del 2008

A Elsie, Oswaldo, Christian y Juan Sebastian…

RESUMEN

El daño cerebral por asfixia es un problema neonatal común y una de las principales causas de muerte en este grupo etario. Además, puede generar trastornos multisistémicos inmediatos y largo plazo. Su daño esta causado por una reducción el la circulación útero-placentaria y su concomitante déficit nutricional, isquemia y trastorno en el intercambio de gases.

El conocido fenómeno de precondicionamiento hipóxico-isquémico se observa en varios sistemas, en los cuales, episodios breves de de isquemia no letal inducen protección contra otro nuevo evento isquémico mayor y letal.

El presente estudio se realiza con el fin de investigar el efecto neuroprotector del precondicionamiento hipóxico-isquémico, específicamente en corteza prefrontal cerebral. El modelo utilizado en ratas, consistió en provocar un evento asfíctico leve al bloquear durante 30 minutos, las arterias uterinas y ováricas de una ratas que cursan un embarazo. Luego, de los diferentes grupos de estudio, las ratas bebe con o sin asfixia fetal fueron sometidas a un evento asfíctico perinatal severo al sumergir el útero de las ratas embarazadas en un baño de agua por 18 minutos.

El efecto de precondicionamiento en la corteza cerebral fue analizado por estimaciones volumétricas de material neuronal y por la proporción de células apoptoicas presentes, en los diversos grupos de estudio.

Como resultado, se evidencio menor apoptosis en el grupo con precondicionamiento, comparado con el que no fue sometido a precondicionamiento. Por lo tanto, el amelioramiento de la apoptosis es probablemente uno de los mecanismos por los cuales se da el fenómeno de precondicionamiento.

ABSTRACT

Asphyctic brain injury is a common neonatal problem and a leading cause of death in this age group. It also has severe immediate and long term multisystemic consequences on the newborn. It's caused by a reduction in the uterine or placental blood flow and the ensuing nutrient deficit, ischemia and gas exchange impairment to the infant.

Hypoxic-ischemic preconditioning is a well known phenomenon, observed in different tissues, in which, brief episodes of sublethal ischemia induce robust protection against the subsequent lethal ischemia in many organs.

The present study is performed to investigate the neuroprotective effect of hypoxic-ischemic preconditioning in the cerebral prefrontal cortex. The model used in rat pups consisted in provoking a mild asphyctic insult, by clamping the uterine and ovarian arteries of a pregnant dam for 30 minutes. Then, pups with or without fetal asphyxia, were then subjected to severe perinatal asphyxia was by immersing the uterine horns in a water bath for 18 minutes.

The effect of hypoxic-ischemic preconditioning was analyzed by volume estimates and a proportion of apoptotic cells in the brain matter after the insult in the different study groups.

As a result, there was a lower amount of apoptosis in the preconditioned study group compared to the group that didn't undergo preconditioning. Therefore, downregulation of apoptotic cell death is probably one of the mechanisms involved in hypoxic-ischemic preconditioning.

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INTRODUCTION:

Acute perinatal asphyxia (PA) or hypoxic-ischemic (HI) injury is a serious neonatal problem. It has detrimental consequences on several organs in the infant (brain, heart, lungs, gut, kidneys), and is a cause of limitating disabilities such as: metal retardation, cerebral palsy and seizure disorders. In this context, HI-preconditioning is a phenomenon that has caught attention for researchers. It involves the induction of a sublethal HI insult that generates a protection effect to a subsequent lethal insult that correlates to that of acute perinatal hypoxia-ischemia.

Strackx *et al.* evaluated this phenomenon based on measurements of apoptosis in the cerebral striatum of rat pups. Results state that this effect lowers the amount of apoptosis caused by a perinatal insult[30]

The present study was conducted to evaluate whether HI preconditioning prevents or modulates the impact of perinatal asphyxia on the prefrontal cortex of the rat brain.

PERINATAL ASPHYXIA:

Definition:

Perinatal asphyxia (PA) occurs when the fetus does not receive enough oxygen before, during or just after birth. Prolonged hypoxia-ischemia is associated with a wide variety of organ damage, particularly brain damage, such as: transient ischemic attack, brain infarction, brain edema, coma and ultimately death. Hypoxic-ischemic encephalopathy (HIE) is a disorder caused by this reduction of oxygen in the blood (hypoxia), combined with reduced blood flow (ischemia) to the brain from a localized obstruction of a cerebral artery or from systemic hypoperfusion. Several variables (clinical and biochemical) are considered to characterize HIE. Criteria defined by the American Academy of Pediatrics (AAP) and the American College of Obstetricians and Gynecologists (ACOG) summarize factors that define potentially moderate to severe hypoxic-ischemic encephalopathy **[Table 1]**:

Table 1: Factors defining potentially moderate to severe hypoxic-ischemic encephalopathy. All of the following must be present for the designation of asphyxia in a newborn[1].

- Profound metabolic acidemia (pH <7) on an umbilical cord arterial blood sample.
- Persistence of an Apgar score of 0 to 3 for >5 minutes.
- Clinical neurologic sequelae in the immediate neonatal period to include: seizures, hypotonia, coma or HIE.
- Evidence of multiorgan system dysfunction in the immediate neonatal period (at least one):
  - Pulmonary: pulmonary hypertension, surfactant disruption, meconium aspiration.
  - Renal: oliguria, acute renal failure.
  - Cardiovascular: tricuspid insufficiency, myocardial necrosis, shock/hypotension.
  - Metabolic: metabolic acidosis, hypoglycemia, hypocalcemia, hyponatremia.
  - Gastrointestinal: necrotizing enterocolitis, hepatic dysfunction.
  - Hematologic: thrombocytopenia, disseminated intravascular coagulopathy.
  - Death.

Acidemia is a key component when discussing asphyxia. As the fetus becomes hypoxic, it also becomes more dependent on anaerobic glycolisis. Therefore, glucose can only be oxidized to pyruvate and lactate. Moreover, there's a buildup of CO_2 in the circulating blood. The accumulation of these acids and CO_2 cause the fall in pH and the ensuing metabolic/respiratory acidosis.[36]

Epidemiology:

In general, perinatal pathologic and traumatic conditions account for 60% of all neonatal deaths and for 33.7% of morbidity rates in the United States[32]. Perinatal asphyxia though, is a worldwide problem. For an estimation of all 4 million neonatal deaths that occur worldwide each year, the three principal causes are: preterm birth, birth asphyxia and infections[17].

Current data suggest that between 2 to 5 of 1000 full-term newborns in the United States, and more so in developing countries, suffer PA at or shortly before birth. Approximately 20% to 40% of those infants, who present HIE, die during the newborn period. Moreover, Lawn *et al.* estimated that a 23% of all neonatal mortality, from any cause was attributed to asphyxia. This represents 940.000 deaths in the year 2000[16] **[Figure 1]**. For the rest that survive, about 25% will exhibit brain injury showing significant neurological and developmental impairments and 10% will show permanent brain damage[2,35]. This condition is more prevalent in preterm infants (60-73%) and is associated with low birth-weight in newborns[30,32].

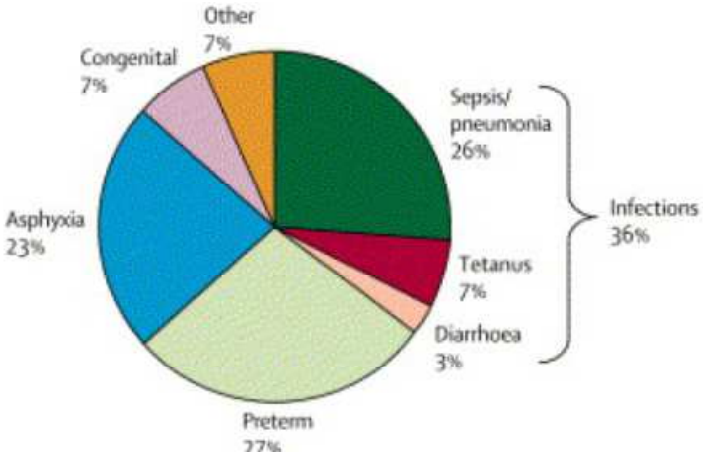

Figure 1: Estimated distribution of direct causes of 4 million neonatal deaths. Proportions estimated for 145 countries for the year 2000[17]. (Adapted from Lawn *et al., 2005*)

Causes:

Perinatal Asphyxia occurs primarily as a result of an acute reduction in the uterine or placental blood flow and the ensuing nutrient deficit and gas exchange impairment[4,36]. Even though the fetus has the capacity to reduce overall oxygen consumption and protect vital organs (brain, heart) by redirecting cerebral blood flow (CBF), acute injury takes place when the severity of asphyxia exceeds this capacity. This aptitude of the fetus is achieved by several mechanisms [**Table 2**]:

Table 2: Mechanisms for fetal blood flow redistribution during hypoxia-ischemia.

· Cerebral vasodilatation.
· Increase of peripheral vascular resistance, by:
– Increased sympathetic activity (adrenaline, noradrenaline).
– Increased chemoreceptor activity.
– Release of arginine vasopressin (AVP).

In addition, some other mechanisms help the fetus to cope with the initial asphyctic insult. Glucose can be released into circulation to increase it's availability to vital regions during asphyxia[36]. Moreover, suppression of cerebral electrical activity mediated by inhibitory neuromodulators (adenosine, GABA, opiates) happens in order to cutback on oxygen consumption. The later is evidenced by a slowing or a shift towards low frequency brain activity seen in the electroencephalogram (EEG)[3].

Overall, five principle mechanisms for PA have been described in the human infant during labor, delivery and the immediate postpartum period **[Table 3]**:

Table 3: Mechanisms of asphyxia in the human infant[6].

· Interruption of the umbilical circulation (compression or accidentated cord).
· Altered placental gas exchange (placental abruption, previa, insufficiency).
· Inadequate perfusion of the maternal side of the placenta (maternal hypotension, hypertension from any cause, abnormal uterine contractions).
· Impaired maternal oxygenation (cardiopulmonary disease, anemia).
· Failure of the neonate to accomplish lung inflation and successful transition from fetal to fetal to neonatal cardiopulmonary circulation.

Pathophysiology:

Various cellular mechanisms and chemical pathways are involved in the damage caused by the hypoxic-ischemic insult. Some of them may arise at the very event of the insult, while others may occur later on. To understand this, it's important to know the sequential progression of events that take place in the brain following hypoxia **[Figure 2]**:

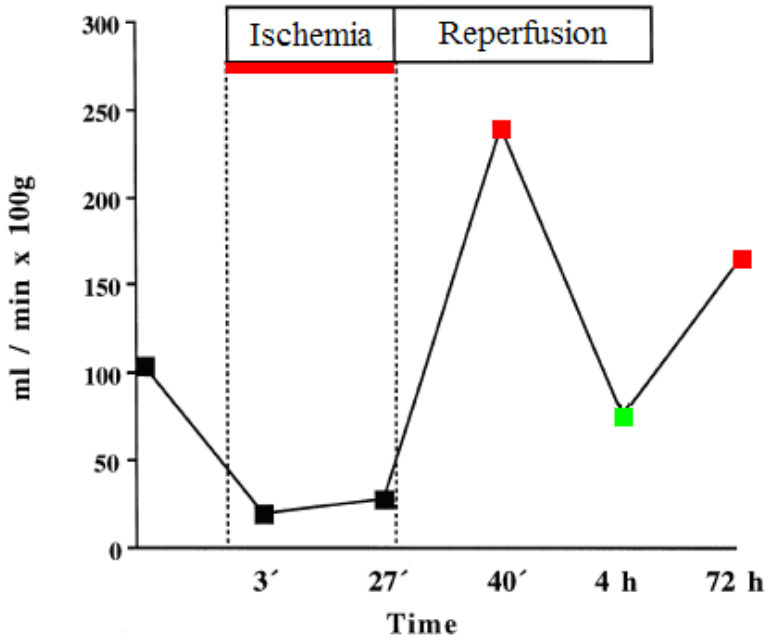

Figure 2: Cerebral blood flow after ischemia. Blood flow undergoes periods of hyperperfusion (■) and a period of hypoperfusion (■) after severe ischemia[4]. (Adapted from Berger *et al.*, 1999)

Initially, the fetus reacts to oxygen deprivation by redirecting blow flow as previously mentioned. Next, lowered P_{O2} and increased P_{CO2} leads to cerebral vasodilatation and a hyperperfusion momentum takes place, mostly on the brainstem. Later, a phase of hypoperfusion ensues that may be related to newly formed oxygen radicals. Shortly, another phase of hyperperfusion takes place. Reperfusion is clinically characterized by a transient period of tachycardia and hyperemia. Interestingly, in cases of severe ischemia, failure of reperfusion to various areas of the brain may happen. This is known as "no-reflow phenomenon" and is related to an increased viscosity of the blood, brain edema and compression of the smallest blood cells[4,36].

During hypoxemia, the fetus maintains cerebral metabolism in an anaerobic fashion. The reduced amount of ATP cause malfunctioning of the Na^+/K^+ pump, which in turn affects these ion gradients, also including Ca^{++}. The membrane potential diminishes causing more ion influx, specially Ca^{++}, and a subsequent swelling of the cell or "cell edema"[4].

Glutamate is known for playing an important role in the pathophysiology of asphyxia as well. In neurons, it activates postsynaptic receptors (AMPA, NMDA and KA), which drive the influx of Na^+, K^+ and Ca^{++}. Lack of energy due to ischemia and the absence of a proper membrane gradient cause a increased release and impaired uptake of glutamate[30]. On the other hand, it activates metabotropic receptors (i.e. quisqualate) that regulate G-protein signal cascades that ultimately cause an increase of free Ca^+ in the cell's cytoplasm (calcium overload). It's been suggested that two waves of glutamate release occur, one during ischemia and another one during the reperfusion phase[4].

Calcium overload, as a result of different mechanisms already mentioned, may cause cell damage by activating proteases, lipases and endonucleases. Also, it is considered to cause alterations in the arachidonic acid cycle, disturbances in gene expression and protein synthesis, and a increased production of free radicals[4,30]. It is one important mechanism during primary cell death (see Apoptosis below)[30].

There is also inhibition of protein synthesis, both during and early after ischemia. This can be related also to a disturbance in Ca^{++} homeostasis in the endoplasmic reticulum. Moreover, it causes dissgregation of polyribosomes, especially in the post-ischemic period and a restitution

of protein synthesis later on. But this new protein synthesis is associated to an apoptotic process[4].

The secondary cell damage occurs during reperfusion. It's driven by pathways which involve generation of reactive oxygen radicals, nitric oxide (NO), inflammatory reactions and excitatory aminoacids like glutamate[4].

The generation of free oxygen radicals during reperfusion can cause membrane damage in most susceptible organs (brain, heart, lungs)[36]. The cutback in oxidative phosphorylation by ischemia produces considerable amounts of adenosine and hypoxanthine which later will be metabolized to xanthine and uric acid. Due to unregulated activity of xantine oxidase during ischemia, a large amount superoxide radicals and then hydroxyl radicals are generated. Activated inflammatory cells produce oxygen radicals as well[4]. Reactive oxygen species are known for triggering mitochondrial dysfunction, causing permeabilization of the mitochondrial membrane and leakage of components, such as cytochrome C[30].

Nitric Oxide also increases the toxicity caused by superoxide radicals by converting them to other highly potent radicals that cause cell damage. Nitric Oxide production is NO-synthase driven. Levels of this enzyme rise, stimulated by the massive Ca^{+} influx during ischemia, and also by cytokines which make inflammatory cells produce it[4].

Lastly, inflammatory reactions are involved in the pathogenesis of this condition as well. Expression of cytokines such as: IL-1, IL-6, TGF-β and fibroblast growth factor. Increased

expression of adhesion molecules like P-selectin, E-selectin and ICAM-1 has been observed as well. As mentioned before, this path leads to further production of damaging oxygen radicals.[4]

It is important to recognize that most of these mechanisms are linked between each other, and it appears that one important end point is the Ca^{++} overload, which ultimately leads to cell apoptosis.

Consequences:

Hypoxic-ischemic brain damage is evolving process that begins during the insult and continues further into the recovery period due to delayed cell death[30,35]. Under severe asphyctic conditions (blood oxygen content <1mmol/L), the fetal protective mechanisms described above are overwhelmed. The immediate consequences are: the failure of redistribution of blood flow to vital organs and the upholding of cerebral oxygen delivery. If the insult is severe enough, cardiac asystole may ensue and eventually death[36].

Brain injury, on the other hand, is strongly associated with the loss CBF due to cardiac output decline[36]. It's also associated to the degree of acidemia, hypoxia and hypercarbia[30]. Immediately after a severe asphyctic episode, a phase of neurologic depression may develop, characterized by hypotonia and suppressed EEG. Also common are post-asphyxial seizures that may last more than 30 min and are related to bad prognosis[36].

The spectrum of damage after an asphyctic insult is broad. As mentioned before, is possible that the fetus might die as a consequence of the insult. What's more, it might recover with no evident neurological damage. In between, the most common outcome is that the fetus might survive with short-term neurological and/or behavioral changes[30]. These include: cerebral palsy, mental retardation, seizure disorders, dystonia, spastic paresis, choreo-atetosis, ataxia, learning and memory deficits[4,5,10,22,30]. Since a great proportion of asphyctic children is born before term, an common consequence is spastic diplegia and hemiplegia, due to periventricular white matter necrosis often seen in this group[36].

Management/Treatment:

Despite numerous research has been done on the subject, practical methods to reduce ischemic brain injury in a clinical setting have not been well established yet. Management of acute PA include: correction pH and glucose levels, blood pressure stabilization, seizure control, maintenance of normothermia, ventilation assistance and resuscitation[19,30]. Also, pharmacologic treatment may be attempted in order to reduce cerebral edema, by using: glucocorticosteroids, osmotic diuretics or barbiturates[35].

Due to the two-phase damage that occurs (ischemic insult and reperfusion phase), and because delayed cell death is present later on (days), there’s a therapeutic window in which intervention might be effective in reducing brain damage. In fact, the first hour after asphyxia represent the most important window, because it’s the hour previous to the final execution of the delayed apoptotic cell death phase[30].

One strategy that has been studied is the use of hypothermia. It's benefits are known in a variety of insults: brain trauma, cerebral hemorrhage, cardiac arrest and seizures[4]. Diverse mechanisms have been attributed to be the foundation its protective effect in the brain: improved recovery of energy metabolism, a cutback on the release of excitatory aminoacids, reduced oxygen radical formation and stabilization of the blood brain barrier or modification of enzyme activity[4]. When studied with perinatal asphyxia models, hypothermia has shown to be able to reduce mortality and long-term cognitive and motor sequelae[19,35,37]. It's the only treatment for which three randomized clinical trials had been conducted, that show improvement of some of the asphyctic infants[11,14,27].

Other strategy still on research is the early destruction of oxygen free radicals generated during and after ischemia. This might be achieved by the administration of specific enzymes that degrade these highly reactive compounds, or by using drugs that inhibiting it's precursors (i. e. allopurinol, oxypurinol)[35].

Since glutamate cytotoxicity plays a key role in the pathophysiology of asphyxia, other therapeutic aim involves inhibition of glutamate release and the blocking of its postsynaptic action. From this therapeutic line, promising drugs include: magnesium sulfate, NMDA and AMPA antagonists[35].

Other drugs still under development include calcium channel blockers that have undergone investigation with no better results than other drugs and inhibitors of NO production[37]. Monosialogangliosides (i.e. GM_1) and growth factors are less studied drugs[35,36].

APOPTOSIS:

As a result of a HI insult, two types of cellular death might take place: necrotic and apoptotic. Necrosis occurs depending on the severity of the insult. Thus, the more prolonged the ischemic period is, the more necrosis that will appear[4]. Interestingly, several studies have demonstrated that apoptosis occurs in brain tissue, also during a prolonged period of time after ischemia[30,34,39].

Apoptosis, also known as "programmed cell death", may be defined as a regulated process leading to cell death via a series of well-defined molecular and morphological changes. Dying cells shrink, condense and then fragment, releasing small membrane-bound apoptotic bodies, which generally are phagocytosed by other cells **[Figure 3]**. As oppose to necrotic cell death, the intracellular constituents are not released into the extracellular milieu where they might have deleterious effects on neighboring cells. The effectors of cell death are cysteine proteases, called caspases. Once pro-apoptotic proteins promote caspase activation, or there's a lack of growth factors, these proteases cleave specific intracellular substrates leading to the demise of a cell[18].

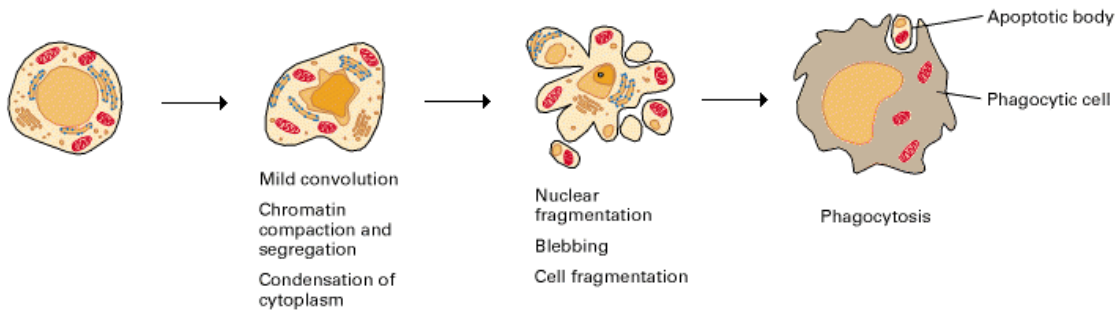

Figure 3: Structural features of cell death by apoptosis. Schematic drawings illustrating the progression of morphologic changes observed in apoptotic cells. Early in apoptosis, dense chromosome condensation occurs, Later both the nucleus and cytoplasm fragment, forming apoptotic bodies. These are phagocytosed by surrounding cells[18]. (Adapted from Lodish. *et al.*, 2000)

Apoptosis after hypoxia has been found in various brain structures: hippocampus, striatum, cortex and cerebellum as a result of an asphyctic event[4,10,30]. Evidence of this process starts as early as 24 hours after the hypoxic-ischemic insult, by measurement of caspase-3 activation; until postnatal day 15 (P15), peaking at postnatal day 8 (P8)[10,30,34].

In the timeline of these pathophysiological changes, four phases can be summarized: insult, reperfusion, latent and secondary phase **[Figure 4]**. The first phase represents the hypoxic-ischemic event in which the cerebral energy machinery fails causing "primary cell death". Subsequently, in the reperfusion phase there's a partial recovery of the oxidative and metabolic processes. The next event is a relatively long latent phase in which a normalization of metabolic processes is seen. Eventually, secondary deterioration phase develops approximately 6-16 hours after the insult. This last period is characterized by the generation of events that will ultimately lead to apoptotic cell death[30].

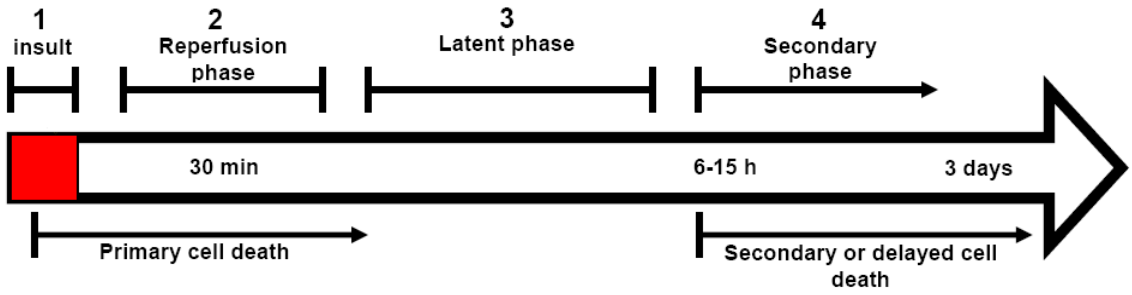

Figure 4: Phases of hypoxic-ischemic injury. Hypoxic-ischemic insult involves 4 phases: ischemic insult, reperfusion phase, latent phase and secondary phase. During the very insult period, the primary cell death process starts. While the secondary or delayed cell death may be evident during the secondary phase[15]. (Adapted from Gunn *et al.*, 2000)

For the apoptosis process to begin, cerebral ischemia induces the expression of a series of proto-oncogenes which code for proteins that might act as transcription factors. Depending on the severity of the insult, these factors are capable of trigger apoptotic cell death[4]. Expression of p53 gene has also been demonstrated. The role of the protein codified by this gene might take the cell to a "conflict in decisions", since it interacts in pathways determined to halt replication and also to induce DNA repair or cell division cycle entry. The conflict subsides when the cell is unable to accomplish any of the latter two. Thus, apoptosis is induced[4]. Moreover, p53 promote Bax-mediated programmed cell death[30].

In addition, during reperfusion, the release of reactive oxygen species is related to mitochondrial dysfunction and the release of cytochrome C to the cytosol. Cytochrome C can trigger the activation of an apoptotic caspase cascade by forming the caspase-9-activating complex. Plus, reactive oxygen species themselves can cause DNA damage and activate p53 too[30].

Interestingly, the delayed apoptotic cell death may be caused by events starting as early as 6 hours after the insult. One theory is that it's the result of a complex apoptotic physiological cascade that may include: accumulation of toxic metabolites (glutamate), overproduction of free radicals, over-expression of Fas death receptor, caspase-8 activation, cytosolic accumulation of cytochrome C[30].

HYPOXIC-ISCHEMIC PRECONDITIONING:

Hypoxic-ischemic preconditioning is a well known event that has been observed in different tissues (nervous, kidney, heart). Initially described by Murry *et al.*, in 1986 in the myocardium, preconditioning is a phenomenon in which brief episodes of sublethal ischemia induce robust protection against the subsequent lethal ischemia in many organs[5,30,39]. Two forms of preconditioning are distinguished: acute and delayed. The first one is short-lived and is mediated by post-translational modification of proteins (i.e. phosphorylation of proteins by certain kinases). The second one is mediated by new protein synthesis and can sustain for days or weeks[9,30]. Even though, several research has been done in this field, the exact mechanisms by which this phenomenon occurs are unclear.

Previous studies, on adult animal models show that periods of sublethal cerebral HI provide protection against a subsequent, more severe insult[5]. Mechanisms suggested for this protection include: up-regulation of glucose transporter-1, reduction in caspase-3 activity, modulation of genomic response with expression of pro-survival inhibitors of apoptosis[29], modulation of protein synthesis, down-regulation of glutamate receptors (GLUT-2)[31] and NMDA[8] and induction of heat shock protein-70 (HSP-70)[8,30,39]. Also, there's evidence that inhibition of inducible nitric oxide synthase (iNOS), a isoform for NO-synthase, abolish neuroprotection against postnatal ischemia[39]. Therefore, NO may be a molecule that plays a double role in this pathophysiology.

Signaling for this process might be NMDA activation dependent **[Figure 5]**. Activation of this receptor causes accumulation of intracellular Ca^{++} and new protein synthesis. First, NO is

produced via activation of nNOS which in turn is coupled to the NMDA receptor by the scaffold protein (PSD-95). Then, NO activates $p21^{RAS}$ (RAS), which will trigger a cascade of kinases: RAF, mitogen-activated protein kinase/extracellular regulated kinase (MEK) and extracellular regulated kinase (ERK). Importantly, inhibition of any of these elements suppresses the tolerance effect. Which proteins are mediating hypoxic-ischemic tolerance at this point are not known[9]. A potential candidate is cAMP-response-element-binding protein (CREB)[12], that is related to the activation of anti-apoptotic genes (i.e. Bcl-2), brain derived neurotrofic factor (BDNF) and insulin. An inducible factor that activates anti-apoptotic proteins, nuclear factor-κB (NF-κB), may be also involved. Finally, hypoxia inducible factor-1 (HIF-1) may be involved in the up-regulation of genes related to angiogenesis, vasodilatation and energy metabolism (i.e. VEGF, EPO) as well[9,30].

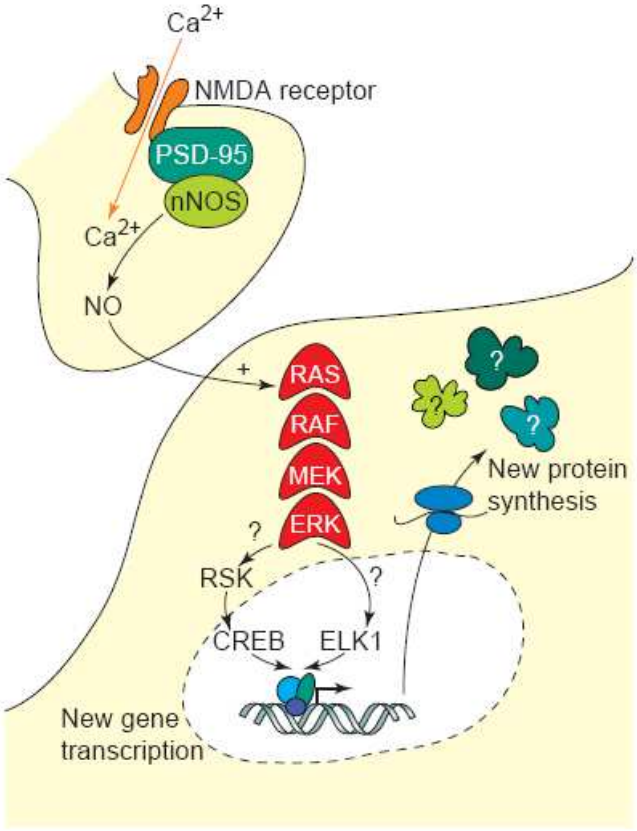

Figure 5: Signal cascade of neuronal preconditioning. Activation of the NMDA receptor leads to Ca^{++} influx and nNOS dependent NO production. NO triggers a kinase cascade of RAS, RAF, MEK and ERK. Proteins that mediate the preconditioning phenomenon are unknown, but CREB and ELK are attractive alternatives[9]. (Adapted from Dawson *et al.*, 2000)

Interestingly, previous studies showed that this phenomenon occurs not only in the adult rat, but also in the immature ones[5]. An hypoxic event (8% oxygen for 3 hours) was able to develop a preconditioning effect with minimal or non brain injury from a second stroke fashion insult later on, in 7-day old pups[13]. Also, reduction of infarct size and increased neuronal survival has been documented[5]. Decreased apoptosis has been demonstrated as well, by measuring less caspase-3 activation[37].

Preconditioning on the immature brain seems to be related to additional mechanisms. In this setting, tolerance seems to be dependent on the synthesis of new RNA[28]. Therefore, increased trasncription found, that might be related to it may be from VEGF, EPO and GLUT-1. On the other hand, the NMDA receptor activation and NO production are also involved[37]. This tolerance effect lasts for a lapse between days, up to one week[5,39].

PREFRONTAL CORTEX AND STRIATUM:

The prefrontal cortex has been implicated in planning complex cognitive behaviors, personality expression and moderating correct social behavior. Its function is role termed "executive function". It implies the ability to differentiate between conflicting thoughts (i.e. differentiate between good and bad), future consequences of current activities, working toward a defined goal, prediction of outcomes, expectation based on actions, and social control[21]. In this function it's also closely related to the striatum.

In the cerebral cortex, damage after an HI insult causes region specific injury, termed "selective neuronal cell death"[4]. Not much research has been conducted on preconditioning in the prefrontal cortex. Evidence of a hypoxic tolerance effect has been demonstrated on the prefrontal cortex by Maruoka *et al.* using 3-nitropropionic acid (3-NPA) in adult rats. Interestingly, a further study measured cerebral glucose metabolism rate in rats pretreated with 3-NPA and found region specific metabolic recovery, suggesting that the tolerance effect may have different mechanisms depending on which part of the brain is triggered[20]. Also, expression of TNF and IL-1β are linked to ischemic tolerance in the setting of a preconditioning model[23]. Therefore, more investigation should be conducted.

On the other hand, Strackx *et al.* studied the HI preconditioning effect in the striatum of 8-day old pups by means of TUNEL staining and stereological analysis for volume estimates of cerebral tissue. Also, other variables were observed, such as: mortality and differences in body weight; at birth, P8 and P15[30]. Results for this study showed less striatal apoptotic cell

death in the preconditioning group, implying that fetal HI preconditioning provides a robust neuroprotection against severe PA.

The present study is preformed to evaluate whether HI preconditioning prevents or modulates the impact of perinatal asphyxia on the prefrontal cortex of the rat brain using the animal model previously developed by Strackx *et al*. Our hypothesis is that subjects under a preconditioning effect, caused by a mild fetal asphyctic insult before birth, sustain a reduction in apoptosis and in the amount of lost brain tissue in the cerebral prefrontal cortex after a severe perinatal asphyctic insult, compared to subjects that undergo only the perinatal insult.

MATERIALS AND METHODS:

ANIMAL MODEL:

For this study, pregnant Wistar rats (Charles River, Maastricht, Netherlands) were delivered on day 14 of gestation. They were housed individually at the University of Maastricht facility for experimental animals. The animals were kept under standard laboratory conditions (21±2˚C ambient temperature, a 12-h light/dark schedule, background noise provided by a radio, and food and water *ad libitum*)[30].

The model was developed as a combination of two previous models. One of mild fetal asphyxia (mFA) used as the first hit at the embryonic day 17 (E17)[5] and one of severe perinatal asphyxia (sPA) used as second hit at E21 or E22[33]. Four different groups were obtained **[Table 4]:**

Table 4: Experimental groups and interventions. Four different groups were obtained: control cecarean delivery (CCD), mild fetal asphyxia (mFA), severe perinatal asphyxia (sPA) and mild fetal asphyxia combined with severe perinatal asphyxia) mFA+sPA.

Experimental Group	Intervention		Abbreviation	Number of subjects
	mFa	sPA		
Control Cesarean Delivery		E21/22	CCD	4
Mild Fetal Asphyxia	E17		mFA	4
Severe Perinatal Asphyxia		E21/22	sPA	3
Mild Fetal Asphyxia + Severe Perinatal Asphyxia	E17	E21/22	mFA+sPA	4

Pregnant rats were randomly assigned to an experimental group: mFA (n=11), sPA (n=7), mFA+sPA (n=11) and CCD (n=7). Only male offspring were used because morphological and behavioral data show a much remarkable impact of asphyxia in male rats[19] and also, because there's probably a protective role of circulating estrogens in females[38]

Since the rat is a postnatal brain developer, the study is conducted at P8 instead of immediately after birth. This way, the results could be more accurately extrapolated to humans. The stage of neuronal development of a term rat can be compared to a preterm to very preterm human baby. In this case, the cerebral developmental stage of a 7 day old pup, could be compared to a full term human baby.[30]

Mild Fetal Asphyxia:

For the mFA insult, the pregnant rats were anaesthetized at E17 by means of isoflurane (4% induction; 2% maintenance). A controlled environment was used (37˚C, 60%-80% humidity, room air) to prevent hypothermia. Then, rats were subjected to a midline laparotomy and the uterine horns exposed. mFA was achieved by clamping uterine and ovarian arteries bilaterally with removable clamps for 30 minutes **[Figure 6]**. During this period, uterine horns were kept wet by rinsing them with saline solution. After 30 minutes the clamps were removed, uterine horns placed back in the abdominal cavity and the abdominal wall was repaired. Later, for recovery, the rats were placed in an incubator for 30 more minutes[30].

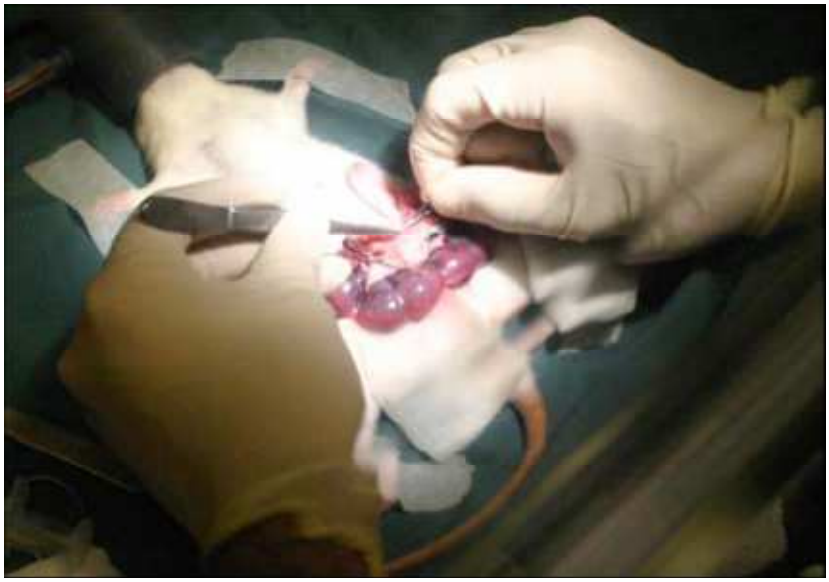

Figure 6: Fetal Asphyxia. Uterine horns were exposed by laparotomy and uterine and ovarian arteries were clamped for 30 minutes[30]. (Courtesy of Strackx E., 2006)

Severe Perinatal Asphyxia:

For the sPA insult, 18 pregnant rats at E21 or E22 (11 with previous mFA and 7 without) were euthanized by decapitation and hysterectomized. Uterine horns were removed and one of them in every case was submerged in a water bath (37˚C) for 18 minutes **[Figure 7]**. Afterwards the uterine horn was opened. The pups were removed, cleaned and stimulated manually to breathe inside a neonatal closed incubator (37˚C, 60%-80% humidity, room air). Afterwards they were left in the incubator for another 60 minutes. The fetuses from the other horn were delivered immediately by opening the uterus as well and received the same stimulation as mentioned above[30].

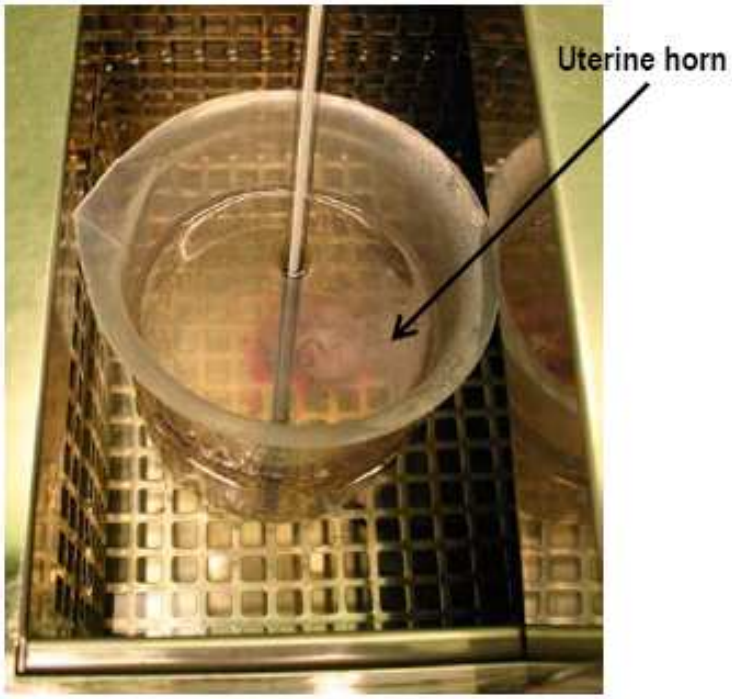

Figure 7: Severe Perinatal Asphyxia. The uterine horns were submerged into a water bath (37˚C) for 18 minutes[30]. (Courtesy of Strackx E., 2006)

Up to two male pups per litter per condition were examined to discard litter effects. The pups were cross-fostered to surrogate dams which have given birth normally the same day. Each dam received between 10 and 13 pups.

TISSUE PREPARATION:

At postnatal day 8 (P8), the pups were anesthetized with sodium pentobarbital (180mg/kg). They were perfused intracardially (30/74rpm), first by a flush of tyrode solution (30s), and later by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.2 M phosphate buffer (pH 7.2). Afterwards, the brain material was removed from the skulls and postfixed for 24h in 4% phosphate buffer. Brain tissue was cryoprotected by immersion in 10% sucrose/0.1M phosphate buffer overnight, followed by immersion in 20% sucrose/0.1M phosphate buffer for 48h; both at 4˚C. Later on, the brains were quickly frozen and stored at -80˚C until further processing. The brain hemispheres were cut into sections on a cryostat (Leica CM 3050); right hemispheres of 30µm-thick for the stereological analysis and left hemispheres of 16µm-thick for TUNEL staining[30].

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling staining)

Cell death is identified by TUNEL staining. Here, terminal deoxynucleotidyl transferase binds to exposed 3'-OH ends of DNA strand breaks, generated principally by apoptosis signals. Apoptosis is further differentiated from necrosis on the basis of characteristic morphology[34]. The staining is described according to Van de Berg *et al.*[34]. As such, sections were washed with TBS, then permeabilized for 10min using Methacarn (methanol:chloroform:acetic acid; 66:33:1) and then rinsed in TBS (3 times per 10min). Subsequently, DNA fragments were labeled with the reaction mixture in a humidified box at

37°C for 1.5h. This reaction mixture consisted of 0.1µl TdT (400U/µl); 1µl cobalt chloride (2.5 mM); 2µl TdT reaction buffer; 0.4µl biotin-dUTP (2 nM); 6.5µl MilliQ (Roche Diagnostics Nederland B.V., Almere, The Netherlands). During incubation, sections were covered with 10µl of the reaction mixture and coverslipped with parafilm. The reaction was stopped by placing the slides in 4x standard saline citrate (SSC) buffer (300 mM sodium chloride, 30mM sodium nitrate, pH 7.5) for 5 min at room temperature, followed by two 5-minute washing steps in TBS. Next, streptavidin-alexa 594 diluted in TBS containing 0.3% Triton X-100 (TBS-T) (1:2000 dilution; for 60 min at RT, Sigma, The Netherlands) was used to visualize nuclei labeled with biotinylated dUTP. After washing in TBS, sections were counterstained with Hoechst 33342 in TBS (1:500 dilution; 30 min at RT; Sigma, The Netherlands). The procedure was finished by washing with TBS for 10 min and enclosure with TBS:glycerol (1:3)[30].

TISSUE ANALYSIS:

TUNEL-stained cells analysis:

Delineation of the prefrontal cortex was defined between interaural 11.20mm (anterior boundary) and interaural 7.20 mm (posterior boundary) **[Figure 8a]**. In a coronal cut, the cortex medial boundary consisted of a line drawn from the dorsal tip of the left brain to the top of the corpus callosum. As a lateral boundary, a line from the ventral tip of the lateral ventricle to the top of the cortex in a specific angle of 45°C was taken [33], according to Paxinos and Watson[24] **[Figure 8b]**.

TUNEL-positive cells were counted in the prefrontal cortex of the left hemispheres from 15 rat pups; 4 pups for each group (CCD, mFA and mFA+sPA), except the sPA group which only had 3 pups. All of them sacrificed at P8. Eight stained slides (Bregma 1.60mm-1.80mm) were analyzed per subject. To detect the presence of positive cells, all slices were examined at a magnification 40x with an Olympus AX-70 microscope (using the counting method described by Van de Berg *et al.*[34]. TUNEL-positive cells were identified by their particular intense red staining and they were counted regardless morphology. An estimation of the total number of positive cells in each rat was made by multiplying the sum of TUNEL-stained cells in all slides by the sampling interval (equal to eight). Also, every TUNEL-positive cell was investigated with the Hoechst 33342 staining to look for morphological characteristics from apoptosis, such as: organized chromatin condensation (pyknotic cells) and/or nucleus fragmentation with preservation of plasma membrane integrity. Results are expressed in mean total number of TUNEL-positive cells per prefrontal cortex; and the number of TUNEL-

positive cells per mm^3. The material was examined by an observer blinded to the group assignment of the sections.

Prefrontal Cortex Volume Estimates:

For the stereological analysis, the right hemispheres of 4 pups for each group (CCD, mFA and mFA+sPA) were used, except the sPA group where only 3 pups were used. All of them sacrificed at P8. The right hemispheres were entirely cut to serial coronal sections (30μm-thick). Sections were mounted on gelatinized glass slides, yielding 8-10 sections per animal. Then they were dried and defatted with a Triton-X100 solution. To visualize neurons, cresyl violet stain was used (0.01%, 11 min), revealing Nissl bodies which appear particularly in the cytoplasm of neurons. Later, the slides were coverslipped with DePeX[30].

Estimations for the volumes of the prefrontal cortex were calculated according to the Cavalieri's principle[7]. It gives an unbiased estimate of the volume of the region of interest by multiplying the sum of the profile areas from all sections, with the distance between them. To do so, a cut through the region of interest is required. Then, the profile areas of the sections through the region of interest are measured by tracing the boundaries of that region on video images displayed on the computer[26]. Later, the profile area is calculated automatically by the computer at a stereology workstation, using a StereoInvestigator software (MicroBrightField, Williston, VT)[30]. Between 6 and 8 slides of brain matter were analyzed per subject. Measurements were performed for three grouped areas (layer 1; layers

2+3+4; and layers 5+6) of the cortex and also one cumulative of the entire prefrontal cortex **[Figure 8b, 8c]**.

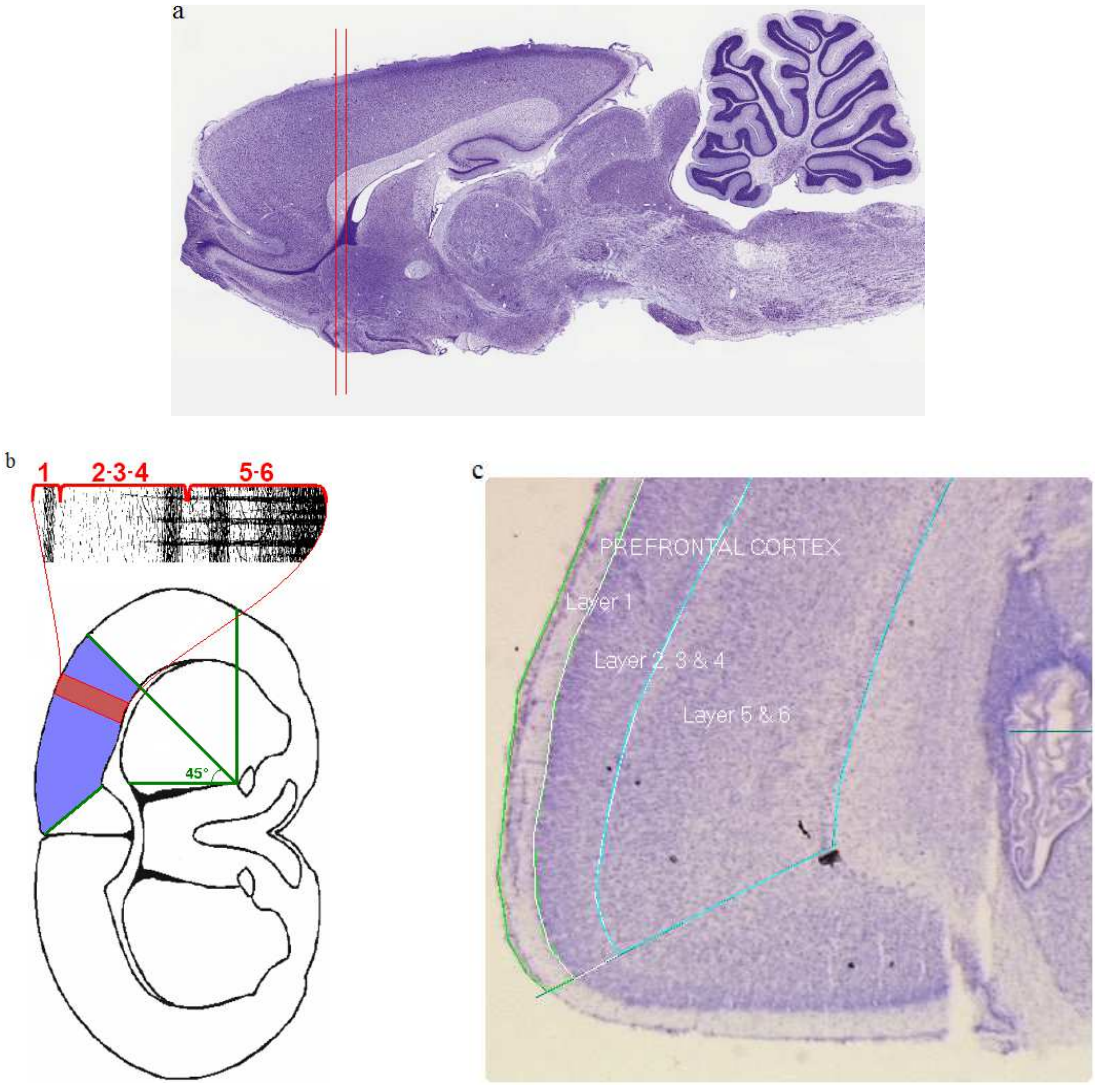

Figure 8: Prefrontal Cortex Delineation and Stereological Analysis. Figure 8 shows sagital and coronal sections of a rat brain. Figure 8a illustrates the prefrontal cortex cut range in a sagital section (note this illustration is not precisely drawn). Figure 8b shows a diagram of a rotated coronal section of a rat brain, presenting the delineation of the prefrontal cortex, along with the different layers in a coronal cut[24,30]. (Adapted from Strackx E., 2006) Figure 8c shows a photography taken with the StereoInvestigator software, showing the same delineation for the volume estimation.

Statistical Analysis:

Data are presented as means, together with standard error of mean (SEM). A two-way ANOVA test was applied to analyze the effect of mFA, sPA, mFA+sPA on the prefrontal cortex volume and the number of TUNEL-positive cells. Differences were considered to be significant at $p<0.05$. All calculations were done using the Statistical Package for the Social Sciences (SPSS 12.0 software).

RESULTS:

TUNEL:

During the observation of TUNEL stained material, apoptotic cell were recognized by their characteristic red staining and also by typical morphological characteristics such as: chromatin condensation, cell shrinkage or fragmentation and presence of apoptotic bodies **[Figure 9]** Particularly, apoptotic cells were mainly found in the first layer of the prefrontal cortex and they seemed to emerge in clusters of various cells.

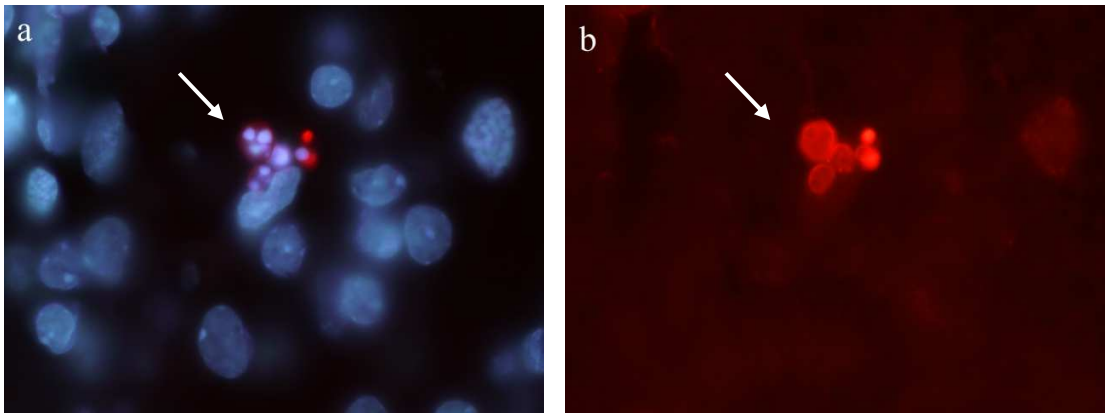

Figure 9: TUNEL staining of the Prefrontal Cortex at P8. Figure 9a shows a fluorescent photography (100x) of Hoechst (blue) and TUNEL (red) staining that contains several normal cells and one undergoing apoptosis (white arrow) evidenced by the presence of apoptotic bodies. Figure 9b shows only TUNEL staining in the same cell.

The results for the estimation of the number of TUNEL-positive cells are presented also as means, together with standard error of means (SEM) **[Figure 10]**:

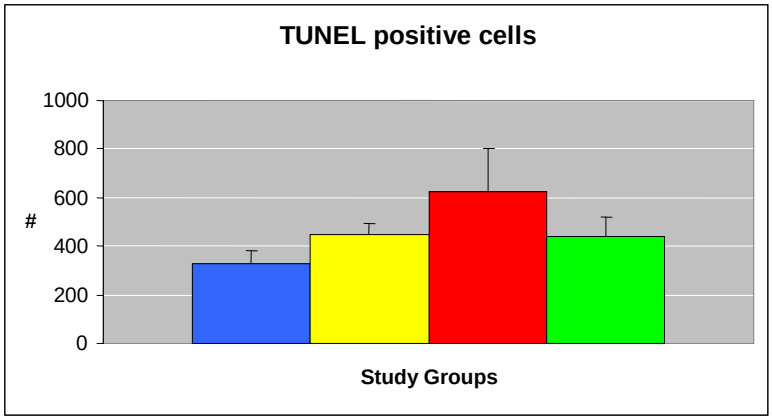

Figure 10: TUNEL-positive cells in the Prefrontal Cortex at P8. Data is expressed as means and SEM of the sum of all layers for the different study groups: CCD(■), mFA(■), sPA(■), and mFA+sPA(■).

There was an increase of apoptotic cells in the three groups (mFA, sPA, mFA+sPA), compared to the CCD group. Also, there is a difference in the magnitude of the effect that mFA caused, compared to the more robust effect that sPA had. These results did not reach statistical significance, although the number of apoptotic cells in the preconditioned group (mFA+sPA) is far less than the number from the sPA group.

VOLUME ESTIMATES:

To evaluate the effect of mFA and sPA in the amount of brain tissue from the different groups, volume estimates were calculated for each layer group (layer 1, layer 2+3+4 and layer 5+6) and also as a cumulative measurement from the prefrontal cortex. All data for volume measurements is presented as means, together with standard error of means (SEM) **[Figure 11]**:

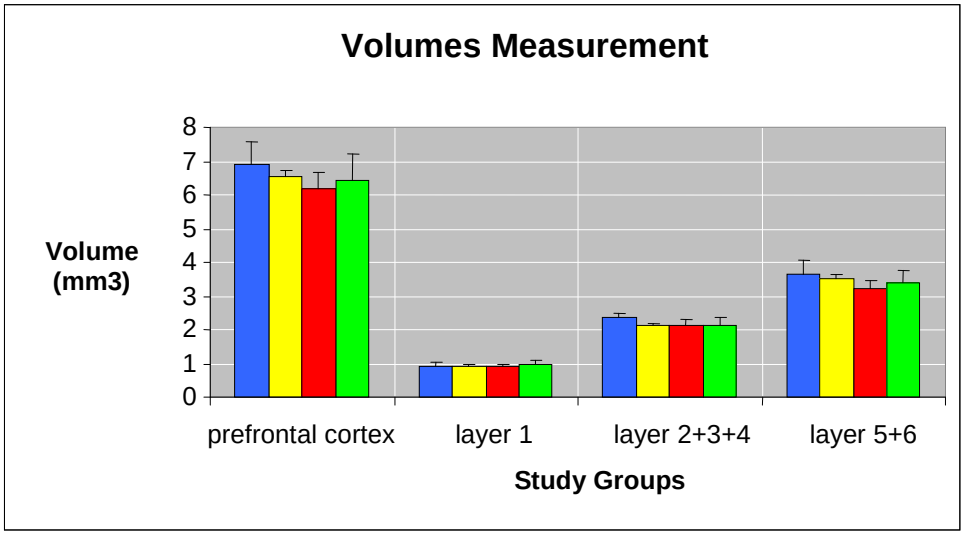

Figure 11: Volume estimates for Prefrontal Cortex at P8: Volumes are presented in mm^3 as means and SEM for the different study groups: CCD(■), mFA(■), sPA(■), and mFA+sPA(■).

Overall, there is a slight difference between groups. Foremost, the CCD group seems to have a modest higher volume proportion than the rest of the groups, while the sPA group has the smallest proportion. A two-way ANOVA analysis to test the effect of mFA, sPA or an interaction of both, didn't reveal statistical significance of the decrease. Neither a post-hoc Bonferroni analysis between groups. Though there's no statistical significance, a tendency to maintain a higher volume is shown by the preconditioning group (mFA+sPA) compared to the group that underwent only sPA.

Lastly, to adjust these measurements to the differences in volumes, estimations for density of apoptotic cells are given, using the data from volume measurements and the number of TUNEL-positive cells **[Figure 12]**:

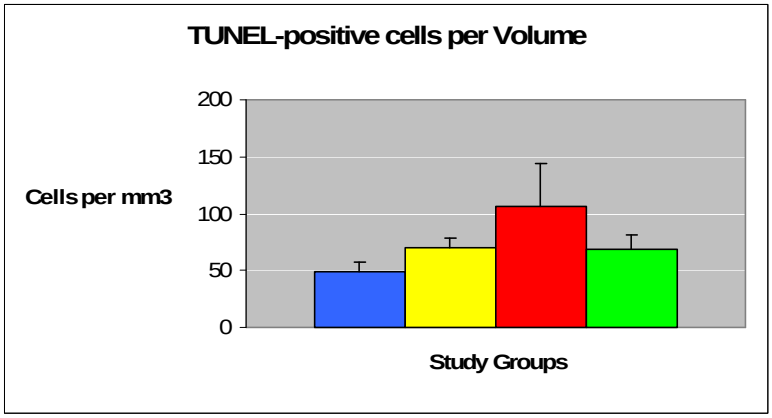

Figure 12: Estimation of the density of apoptotic cells in the Prefrontal Cortex at P8. Data is expressed as means and SEM for the different study groups: CCD(■), mFA(■), sPA(■), and mFA+sPA(■).

The same tendency of apoptosis is also seen here. A two-way ANOVA test didn't find a significant effect of mFA, sPA or an interaction of both. However, post-hoc Bonferroni analysis reveal a significant difference between the CCD and the sPA groups ($p<0.047$). Remarkably, there's a tendency to a lowering in apoptosis outcome due to preconditioning as well.

A frequent and a severe complication of birth asphyxia is death. In our model, 55% of the animals per litter died after a sPA insult of 18 minutes, while none of the mFA or CCD animals died. When the pups had a mild prenatal hypoxic exposure before being exposed to a severe perinatal insult, fewer animals per litter died (39%).

DISCUSSION:

In this study, a fetal HI preconditioning provides protection to apoptotic cell death caused by a severe perinatal HI insult in the prefrontal cortex. This was evidenced by a lower amount of apoptosis in the preconditioned group (mFA+sPA) compared to the group that didn't undergo preconditioning (sPA). Interestingly, all groups show some degree of apoptosis, even the control group (CCD). This corresponds to a physiological cell death in this stage of development. Also, a cesarean surgery sham effect could cause some degree of injury to all of the subjects.

These results correlate with the outcome obtained by Strackx *et al.* in the striatum. Using the same animal model, this study showed less striatal apoptotic cell death in the preconditioning group. Although there's information showing a tolerance effect in the prefrontal cortex, as evidenced by the study by Maruoka *et al.*, in which a chemical inhibition of cell metabolism triggers a neuroprotective effect to hypoxia-ischemia in neuronal tissue[20], no other previous studies of preconditioning evidenced reduction of apoptosis following a hypoxic insult in the prefrontal cortex.

The fact that this model is a global HI insult is critical to differentiate it from previous ones that have been studied extensively in adult pups[25] and later evolved to neonatal models[5,33]. Rice *et al.* conducted a study in which a first ischemic insult was induced at P8 and later, an second hypoxic insult of 3.5 hours. The results showed edema and necrotic changes in the rat brain, but since the first insult was unilateral (unilateral carotid ligation) a

real assessment of the whole brain anatomy couldn't be done[25]. Later, Cai *et al*. demonstrated an hypoxic tolerance effect on a combined HI model, by first causing an global HI insult in utero and subsequently an unilateral neonatal one[5]. Our model demonstrates neuronal effects in a global HI model.

It is also important to note that the hystopathological studies are done at this age (P8), since the rat is a postnatal brain developer. The developmental stage of a 7-day old cerebral cortex is similar to that of the full term baby[5].

Current advances in obstetric and neonatal care have led to a great reduction in neonatal morbidity and mortality. Even though, a structured and highly effective therapeutic approach against hypoxic-ischemic damage is still to be elucidated. This study contributes with more understanding of an intrinsic protective mechanism that is still to be clearly defined and consequently aimed towards a potential therapy. Revealing molecular pathways, by which this phenomenon occurs, are of outmost importance. Theories such as the previously mentioned by Dawson *et al.*[9], are great candidates. This way, a genetic or pharmacological therapeutic approach could be developed. Moreover, is also important to determine if this protective mechanism has a long term impact on behavioral and neurologic function.

It is also important to consider that, as a normal occurrence during pregnancy, the unborn baby could experience very mild periods of asphyxia. For instance when the mother exercises, or when she lives in high altitudes. This could provide a protective effect to the fetus during perinatal asphyxia as well.

In conclusion, using this model of global fetal HI preconditioning we were able to reproduce a protective effect previously seen in the striatum. Even if the effect seen was not strong, reproduction of this experiment using a larger number of subjects will increase the power and render reliable results.

On the other hand, the mechanisms by which this phenomenon occurs are still less clear. Therefore, more studies in this field should aim to uncover these and also to evaluate region-specific differences in molecular and biochemical response to HI.

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GLOSSARY:

AAP	American Academy of Pediatricians
ACOG	American College of Obstetricians and Gynecologists
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid
AVP	arginine vasopresin
BDNF	brain derived neurotrophic factor
CBF	cerebral blood flow
CCD	control cesarean delivery
E	embryonic day
EEG	electroencephalogram
FA	fetal asphyxia
GABA	γ-aminobutiric acid
HI	hypoxic-ischemic
HIE	hypoxic-ischemic encephalopathy
ICAM	intercellular adhesion molecule
IL	interleukin
KA	kainate
mFA	mild fetal asphyxia
mFA+sPA	mild fetal asphyxia + severe perinatal asphyxia
NMDA	N-methyl D-aspartate
NO	nitric oxide
P	postnatal day

PA	perinatal asphyxia
sPA	severe perinatal asphyxia
TGF	transforming growth factor
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling