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HYPOXIA-ISCHEMIA IN NEONATAL BRAIN: A TRANSLATIONAL APPROACH TO ITS CONSEQUENCES

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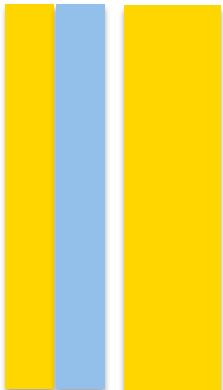

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LIST OF PUBLICATIONS

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Erdi-Krausz G[1], Rocha R[1], Brown A, Myneni A, Lennartsson F, Romsauerova A, Cianfaglione R, Edmonds CJ, Vollmer B. **Neonatal hypoxic-ischaemic encephalopathy: Motor impairment beyond cerebral palsy**. Eur J Paediatr Neurol. 2021 Nov;35:74-81. doi: 10.1016/j.ejpn.2021.10.005.

[1]Contributed equally; first authors.

Leite SS, Matos J, Grenha J, Braga AC, Rocha R. **Neurodevelopmental outcomes of children with periventricular leukomalacia: the role of infection and ischemia**. J Pediatr Neonat Individual Med. 2021;10(1):e100105. doi: 10.7363/100105.

Fonseca M, Rocha R, Garrido C, Braga AC, Frutuoso S, Carvalho C, Proença E. **Ischemic and hemorrhagic perinatal stroke in a neonatal intensive care unit: a 10-years survey**. Archives of Pediatrics and Neonatology, Volume 1, Issue 2, 2018, PP: 1-9

Silva D, Rocha R, Correia AS, Mota B, Madeira MD, Vale N, Cardoso A. **Repurposed Edaravone, Metformin, and Perampanel as a Potential Treatment for Hypoxia–Ischemia Encephalopathy: An In Vitro Study**. Biomedicines. 2022; 10(12):3043. https://doi.org/10.3390/biomedicines10123043

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ABBREVIATIONS

ADHD	Attention-deficit/hyperactivity disorder
ATP	Adenosine triphosphate
GABA	Gamma-aminobutyric acid
HIE	Hypoxic-ischemic encephalopathy
MABC	Movement Assessment Battery for Children
MND	Minor neurological dysfunction
MRI	Magnetic resonance imaging
NMDAR	N-methyl-D-aspartate receptor
NPY	Neuropeptide Y
preOLs	Pre-oligodendrocytes
PVL	Periventricular leukomalacia
TH	Therapeutic Hypothermia
TSHI	Transient systemic hypoxia-ischemia
VPT	Very preterm

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SUMMARY

Hypoxic-ischemic brain injury is caused by the lack of oxygen and deprivation of blood supply and energy. In the neonatal brain, hypoxic-ischemic injury can have different presentations dependent on gestational age, type and severity of the injury. The main goal of this thesis was to explore the neurologic consequences of hypoxic-ischemic injury in the neonatal brain of children born preterm or at term, through a translational approach.

In a retrospective study of children with periventricular leukomalacia, we observed that only about a quarter of children had a normal outcome. Seventy percent of the children had cerebral palsy and 29% had epilepsy. Children with periventricular leukomalacia perinatally exposed to infection may have a higher risk of abnormal developmental outcome or cerebral palsy compared to children exposed to hypoxia-ischemia.

In a review of the outcome of children with perinatal stroke, we showed that neurodevelopment assessment was abnormal in 64% of the cases and motor impairment was present in 41%. We identified at least one risk factor in all cases of ischemic stroke and we saw that the presence of multiple risk factors was significantly associated with a worse outcome.

Regarding hypoxia-ischemia in term children, we reported the clinical outcome at school age of children who suffered neonatal hypoxic-ischemic encephalopathy but did not develop cerebral palsy. About one-fifth of children with neonatal hypoxic-ischemic encephalopathy treated with therapeutic hypothermia had clinically significant motor impairment at school age and this was related to the presence of minor neurological dysfunction. Motor function impairment was associated with general cognitive difficulties and attention problems at school. However, early neurodevelopmental assessment (at 2 years of age) and neonatal brain magnetic resonance imaging were poor predictive markers of school-age neuromotor outcomes and did not correctly identify these children at risk.

In an experimental study, we characterized a rat model of encephalopathy of prematurity in the presence or absence of a prenatal hypoxic-ischemic insult. Prenatal hypoxia–ischemia rats showed delayed development of neonatal reflexes, reduction of anxiety, impairment of learning and memory, and alterations in neurogenesis, NPY-ergic and cholinergic systems. No significant alterations were found in the absence of hypoxia-ischemia. We proposed altered neurogenesis, via an NPY-dependent pathway, as a possible explanation for the memory deficits and reduction in the density of cholinergic varicosities in the hippocampus, as a

reflection of cholinergic basal forebrain dysfunction, and as a possible contribution to the cognitive deficits in children exposed to prenatal hypoxia–ischemia.

Finally, we tested three repurposed drugs (edaravone, perampanel and metformin) to evaluate their capacity to revert or at least attenuate the deleterious effects of hypoxia–ischemia in an in vitro model. Edaravone and low concentrations of perampanel were able to attenuate hippocampal cell damage induced by hypoxia and oxygen-glucose deprivation.

All perinatal hypoxic-ischemic conditions share and embrace a road of life-long disabilities varying from severe cerebral palsy to minor neurological dysfunction. We should be proactive in the identification of these conditions and look for a customized treatment to improve the chance of a normal outcome.

Keywords: Ischemia, Hypoxia, Hypoxic-ischemic encephalopathy, Encephalopathy of prematurity, Periventricular Leukomalacia, Perinatal ischemic stroke

SUMÁRIO

A lesão cerebral hipóxico-isquémica é causada pela falta de oxigénio e privação do fluxo de sangue e energia às células. No cérebro neonatal, a lesão hipóxico-isquémica pode ter diferentes apresentações, dependentes da idade gestacional, tipo e gravidade da lesão. O principal objetivo desta tese foi o de explorar as consequências neurológicas da lesão hipóxico-isquémica no cérebro neonatal de crianças nascidas pré-termo ou a termo, através de uma abordagem translacional.

Num estudo retrospectivo de crianças com leucomalácia periventricular, observamos que apenas cerca de um quarto das crianças tiveram um desenvolvimento psicomotor normal. Setenta por cento das crianças apresentavam paralisia cerebral e 26% tinham epilepsia. Crianças com leucomalácia periventricular expostas à infecção no período neonatal parecem ter um risco maior de desenvolvimento psicomotor anormal ou paralisia cerebral em comparação com crianças expostas a hipóxia-isquemia.

Numa revisão de crianças com acidente vascular cerebral perinatal, mostramos que o neurodesenvolvimento foi anormal em 64% dos casos e existia comprometimento da função motora em 41% dos casos. Identificamos pelo menos um fator de risco em todos os casos de acidente vascular cerebral isquémico e vimos que a presença de múltiplos fatores de risco estava significativamente associada a um pior resultado em termos de desenvolvimento.

Em relação à exposição perinatal à hipóxia-isquemia em crianças a termo, relatamos a evolução clínica, na idade escolar, de crianças que sofreram encefalopatia hipóxico-isquémica neonatal, mas não desenvolveram paralisia cerebral. Cerca de um quinto das crianças com encefalopatia hipóxico-isquémica neonatal tratadas com hipotermia terapêutica apresentavam um comprometimento motor clinicamente significativo e isso estava relacionado com a presença de disfunção neurológica minor. O comprometimento da função motora estava associado a dificuldades cognitivas e a problemas de atenção na escola. No entanto, a avaliação precoce do neurodesenvolvimento (aos 2 anos) e a ressonância magnética cerebral no período neonatal foram fracamente preditivas dos resultados neuromotores em idade escolar e não identificaram corretamente estas crianças em risco.

Num estudo experimental, caracterizamos um modelo de rato de encefalopatia da prematuridade na presença ou ausência de um insulto hipóxico-isquémico pré-natal. Os ratos que sofreram hipóxia-isquemia pré-natal apresentaram atraso no desenvolvimento dos

reflexos neonatais, redução da ansiedade, comprometimento da aprendizagem e da memória e alterações na neurogénese, dos sistemas NPY-érgico e colinérgico. Não foram encontradas alterações significativas na ausência de hipóxia-isquemia. Apontamos a neurogénese alterada, através de uma via dependente do neuropeptídeo Y, como uma possível explicação para os défices de memória, e apontamos a redução da densidade de varicosidades colinérgicas no hipocampo, reflexo da disfunção colinérgica do prosencéfalo basal, como possível fator causal dos déficits cognitivos em crianças expostas a hipóxia-isquemia pré-natal.

Finalmente, testamos três fármacos (edaravone, perampanel e metformina) para avaliar a sua capacidade de reverter ou atenuar os efeitos deletérios da hipóxia-isquemia num modelo in vitro. O edaravone e baixas concentrações de perampanel são capazes de atenuar o dano nas células do hipocampo induzido pela hipóxia e privação de oxigénio-glicose.

Todas as condições hipóxico-isquémicas perinatais compartilham e abrangem um caminho de incapacidades ao longo da vida, variando desde a paralisia cerebral grave a disfunção neurológica minor. Devemos ser proativos na identificação dessas condições e procurar um tratamento personalizado no sentido de melhorar a probabilidade de um desenvolvimento normal.

Palavras-chave: Isquemia, Hipóxia, Encefalopatia hipóxico-isquémica, Encefalopatia da prematuridade, Leucomalácia periventricular, Acidente vascular isquémico perinatal

INTRODUCTION

Neonatal brain is immature, vulnerable and highly energy dependent.

The brain has most of its energy generated by oxidative metabolism since anaerobic metabolism is much less efficient. In the neonate, cerebral oxidative metabolism is thought to play a critical role in the early development of the brain. During gestation, the human brain shifts from anaerobic glycolysis to more efficient aerobic metabolism, in order to meet the high-demand energy requirements of the complex maturational processes[1]. Interruption of oxygen and glucose supply to the neonatal brain is highly detrimental and the clinical consequences are frequently catastrophic and with an extraordinary impact on the quality of life, functionality and social independence of the person.

Hypoxia is a state in which oxygen is not available in sufficient amounts at the tissue level to maintain adequate homeostasis[2], whereas ischemia refers to a state in which blood supply to the tissue is insufficient to maintain cellular normal functioning. Indeed, in clinical practice, hypoxia and ischemia often occur together and potentiate each other. Hypoxia leads to a decrease in cardiac output, which reduces cerebral blood flow, and, on other hand, ischemia originates tissue hypoxia.

Hypoxic–ischemic encephalopathy (HIE) is the medical term used to describe the clinical syndrome and the complex physiological, cellular, and molecular changes resulting from a hypoxic-ischemic event in the brain. In the neonatal period, the incidence of HIE in term neonates is about 1 to 8 per 1000 live births in developed countries and is as high as 26 per 1000 live births in underdeveloped countries[3]. Neonatal HIE is one of the most common causes of death, accounting for 23% of infant mortality worldwide[4].

Hypoxic-ischemic brain injury can affect term and preterm neonates and typically occurs diffusely in the brain, albeit specific regions being preferentially damaged. When it is localized and results from the occlusion of a specific brain vessel, get the name of perinatal stroke.

HYPOXIC–ISCHEMIC ENCEPHALOPATHY IN THE TERM NEWBORN

Basic pathophysiology

The pathophysiology of HIE involves several mechanisms, including glutamatergic excitotoxicity, oxidative stress, mitochondrial energy production failure, and inflammation. The brain is continually dependent on energy in form of adenosine triphosphate (ATP), a process that requires oxygen and metabolization of glucose, lactate, or ketone bodies. In HIE, damage to the brain is initiated by the interruption of blood and oxygen supply to the fetal brain during pregnancy or around the time of birth, in conditions such as chronic maternal hypoxia, pre-eclampsia, umbilical cord knotting, umbilical cord prolapse, shoulder dystocia or placental abruption[5]. Hypoxic-ischemic injury is classically described in three phases: primary, secondary, and tertiary energy failure. In the primary phase of hypoxic-ischemic injury, there is a decrease of mitochondrial phosphorylation, leading cells to switch to anaerobic metabolism, generating much less adenosine triphosphate (ATP) per molecule of glucose[6]. With the reduction of ATP, membrane ionic pumps fail, resulting in the accumulation of intracellular sodium, water, and calcium. This cascade of events leads to cytotoxic edema and depolarization of the cell membrane. The depolarized neuronal membrane releases high concentrations of glutamate causing glutamate receptors overactivation and a sustained influx of calcium into neurons. This generates several deleterious consequences, including mitochondrial dysfunction, reactive oxygen and nitrogen species overproduction, impairment of calcium buffering, activation of lytic enzymes, and release of pro-apoptotic factors[7]. Free fatty acids are also released from degrading cells and peroxidation of free fatty acids by oxygen free radicals occurs, potentiating more cellular damage. Cytotoxic edema, excitotoxicity, oxidative stress, and lipid peroxidation prime the loss of cell membrane integrity and cellular death via necrosis and apoptosis.

Depending on the timing and severity of the injury, a partial recovery occurs during the 30 to 60 minutes after the acute insult. Restoration of blood and oxygen supply to the brain allows the return of aerobic metabolism. However, the reperfusion of the brain also exacerbates brain injury, since reperfusion of ischemic tissues is often associated with microvascular injury and increased permeability of capillaries. During the second phase, which typically extends from 6 to 72 h after the insult, reperfusion leads to the induction of an inflammatory reaction with

microglia activation and subsequent synthesis of reactive oxygen species, mitochondrial dysfunction, and persistent glutamate excitotoxicity[5,8]. During this phase, cerebral autoregulation impairment, hypocapnia-induced vasoconstriction, blood-brain barrier disruption, cerebrovascular endothelium dysfunction with microcirculatory perturbations and microthrombi formation, failure of mitochondrial activity and activation of lytic cellular enzymes contribute to injury[8]. These events can culminate in cell death by necrosis, apoptosis, and autophagy. Microglia migrate to damaged regions and produce inflammatory cytokines, nitric oxide, and free radicals that exacerbate the injury and alter brain repair[9].

In the third phase, which potentially lasts weeks to years, there are persistent anomalous mechanisms that prevent normal repair, causing remodelling and re-organization. During this tertiary phase of brain injury, epigenetic changes and chronic inflammation sensitize the brain to further injury[10]. DNA methylation and active demethylation, histone modifications and microRNAs involved in the regulation of neuronal and vascular developmental plasticity play a role in response to ischemia and hypoxia of the developing brain.

Clinical presentation

Neonatal HIE is a defined clinical syndrome in term infants that results from a severe or prolonged hypoxic–ischemic episode before or during birth. There is no unique test for an accurate diagnosis of HIE in neonates and physicians diagnose it based on the presence of a constellation of a compatible clinical history (risk factors), neurological signs, and results of ancillary studies that support a hypoxic-ischemic insult. Identification of a sentinel event facilitates the diagnosis. Catastrophic asphyxia contributes to approximately 25% of cases of HIE: maternal cardiorespiratory arrest, placental abruption, uterine rupture, fetal entrapment (such as shoulder dystocia), and occasional cases of cord prolapse, cord entanglements and true knots in the cord are red flags to hypoxia and ischemia[11]. Low Apgar scores (5-min Apgar score of 5 or less), and assisted ventilation initiated at birth and continued for at least 10 minutes are also important alerts to HIE[12].

Neurologic dysfunction, in the form of neonatal encephalopathy, is the main clinical presentation, although other organ dysfunctions may coexist. Hallmarks of neonatal encephalopathy include depression of the level of consciousness, often with respiratory depression, abnormality of muscle tone and power, and seizures[5]. In the first hours after a severe ischemic insult, neonates exhibit a depressed level of consciousness and periodic

breathing with apnea or bradycardia. Hypotonia with decreased movement is associated with injury to cortical regions, and seizures may occur in the most severely affected infants very soon after the insult[13]. In a standardized approach, neonatal encephalopathy can be scored according to systems like Sarnat staging or Thompson score[14,15].

Metabolic acidosis strongly suggests hypoxic-ischemic injury and in clinical trials for therapeutic hypothermia (TH), eligibility criteria included a pH of 7.0 or less or a base deficit of 16 mmol per liter or more during the first hour after birth[12]. Injury in other organs also provides further evidence of hypoxic-ischemic injury. Elevated transaminase level, elevated creatinine level, elevated creatine kinase–MB fraction and troponin T levels are indirect evidence of lesion in the liver, kidney, and heart[5].

Brain ultrasound or magnetic resonance imaging (MRI) of the brain can confirm HIE. MRI can help to distinguish HIE from other forms of encephalopathy caused by metabolic and genetic diseases, traumatic or infectious causes. Neonatal HIE is associated with brain injury in the basal ganglia, thalamus, cortex, watershed zones, hippocampus and brainstem, and the pattern of injury is dependent on the severity and duration of hypoxia-ischemia and may correlate with specific neurological impairments. It is important to note that patterns of brain injury emerged with the use of MRI analysis. Indeed, after acute moderate or severe hypoxic-ischemic injury, abnormal signal intensity is commonly detected in the basal ganglia and thalami, corticospinal tract, white matter and cortex. In mild to moderate and more indolent hypoperfusion, abnormal signal intensity is located in the intervascular watershed zones / parasagittal zones.

MRI might be used as a biomarker of the neurodevelopmental outcome. Loss of the posterior limb of internal capsule signal on T1-weighted spin-echo MRI in this region is a reliable indicator of severe hypoxic–ischemic injury and is related to poor motor function[13]. Abnormal MRI findings in basal ganglia and thalami have the greatest predictive value of the adverse outcome and severe motor impairment[16].

Outcome

Prior to the era of TH, disability rates in children with moderate encephalopathy ranged from 6–21%, and in children with severe encephalopathy ranged from 42–100%[17]. Cerebral palsy is infrequent with mild encephalopathy, occurs in about 30% with moderate encephalopathy, and is almost universal with severe encephalopathy[18,19]. Mortality rates are high among infants

with severe HIE, and children who survived neonatal encephalopathy can present cognitive deficits even in the absence of motor impairment.

The NICHD Neonatal Research Network randomized controlled trial of whole-body hypothermia for neonatal HIE noted a mortality rate of 37% in the control group and 24% in the hypothermia group. The rates of moderate or severe cerebral palsy were 30% in the control and 19% in the hypothermia group, with corresponding rates of blindness of 14% versus 7% and hearing impairment of 6% versus 4%[12]. The primary outcome at 6–7 years of age showed that hypothermia resulted in lower death rates and did not increase rates of severe disability among survivors. School-aged survivors of the NICHD Neonatal Research Network randomized controlled trial had subnormal IQ scores in more than a quarter of children.

In the CoolCap trial, at age 18 months, 33% of children in the hypothermia group had died versus 38% in the control group, and 19% had severe neuromotor disability versus 31% in the control group[20]. Follow-up of the CoolCap trial of head hypothermia for neonatal encephalopathy showed that the functional outcome at 7–8 years was associated with 18-month neurodevelopmental assessment[21].

The Total Body Hypothermia for Neonatal Encephalopathy (TOBY) trial evaluated the neurocognitive function of participants at 6–7 years of age with a primary outcome of survival with IQ ≥85[22]. 52% of children in the hypothermia group versus 39% in the control group survived with IQ ≥85. The mortality rate was 29% and 30% in the hypothermia and control groups, respectively. More children in the hypothermia group survived without neurological abnormalities and lower rates of cerebral palsy, and moderate or severe disability than the control group.

Outcome beyond the classical motor picture

Several large randomised controlled trials have shown that TH reduces both mortality and severe neurodisability (cerebral palsy), but less severe impairment continues to appear until school age[17]. Commonly, children are discharged from neurodevelopmental follow-up at 2 years of age if there is no global developmental or severe neuromotor impairment[23]. However, there is evidence that there is still an increased risk for minor neurological, cognitive, or long-term behavioral dysfunctions[24].

Most data about the long-term outcome of children with neonatal HIE who do not develop cerebral palsy come from the period prior to TH. Data from the TH era is limited. A recent study

by Jary and collaborators showed that 38% of school-aged children who survived HIE following TH without cerebral palsy had a subnormal motor function accessed by Movement Assessment Battery for Children-2 (MABC-2) [25]. Also, it was previously shown for a large clinical cohort who underwent TH, that at age 2 years, 12% of children without cerebral palsy had minor neurological signs (gross or fine motor coordination difficulties, muscle tone imbalance, without definite signs of cerebral palsy) and this was associated with poorer motor, cognitive, and behavioral functions compared to those with normal neurology[26]. Minor neurological dysfunction (MND), typically diagnosed at school age, is a description of the neurological profile of a child without cerebral palsy or major impairments, which describes difficulties with posture, muscle tone regulation, balance, fine manipulative ability, mildly abnormal reflexes, movements, coordination, or cranial nerve function[27]. It can be classified as simple or complex MND, depending on the number of dysfunctional domains. Simple MND has limited clinical significance and may represent a normal suboptimal variant of neuromotor development. Complex MND is considered a form of brain dysfunction associated with neuroanatomical deficits and functional impairments such as learning disorders and behavioral problems[28]. Applying the concept of MND to children with neonatal HIE who have not developed cerebral palsy, appears an attractive approach to stratify children's risk and target interventions. In a study by Barnett and collaborators, in children with neonatal HIE not treated with TH, MND and lower motor scores (assessed with Touwen examination and Movement Assessment Battery for Children) were both associated with basal ganglia lesions or focal signal abnormalities in white matter[29]. In contrast, in a study by Perez and collaborators, also in children who were not treated with TH, neonatal MRI pattern did not correlate with motor performance on the Zurich Neuromotor Assessment in children without major disability[30].

HYPOXIC–ISCHEMIC ENCEPHALOPATHY IN THE PRETERM NEWBORN

Basic pathophysiology

Preterm birth is defined as the birth before 37 weeks of gestation and is regarded by the World Health Organization as one of the major health problems. Its incidence in most developed countries is about 5-7% of live births, and approximately one-third to one-half of cerebral palsy

cases result from preterm birth[31]. The neurologic and psychiatric disturbances found in preterm humans are globally explained by encephalopathy of prematurity, a concept introduced by Volpe that is defined by an array of grey and white matter lesions resulting from combined acquired insults, altered developmental trajectories and disorganized reparative phenomena[32]. In preterm, the two main known histopathologic brain lesions responsible for the neurological sequelae are periventricular leukomalacia (PVL) and periventricular hemorrhagic infarction[33]. PVL is characterized by diffuse injury of the deep cerebral white matter around the ventricles and has a deeper zone of focal necrosis and a more peripheral and extensive area where diffuse gliosis predominates. It is characterized by coagulation necrosis, microglial infiltration, astrocytic proliferation, and eventual cyst formation. The ultimate result is the loss of volume of cerebral white matter with secondary ventriculomegaly[34].

The cerebral white matter of the preterm is extremely susceptible to ischemic and infectious insults. Previous data indicate that the pathogenesis of PVL includes the result of the harmful effects of hypoxia, ischemia and inflammation on the progenitor oligodendrocyte cells, which are located in the periventricular area between the 23rd and 32nd weeks of gestation and are particularly vulnerable to such insults[35]. Despite rapid advances in neuroscience research, the available knowledge about the pathophysiology of encephalopathy of prematurity and PVL is still insufficient. An important obstacle to elucidating the pathophysiology of this condition are the limitations of the preclinical models that only partially recapitulate the abnormalities observed in humans[36]. The most commonly used experimental animal models induce brain injury by hypoxic-ischemic and/or infectious insults.

Some factors contribute to the pathogenesis and susceptibility to hypoxia and ischemia in the preterm: 1) the loss of cerebrovascular autoregulation and consequently high-frequency periods of pressure-passive cerebral circulation and inadequate cerebral blood flow; 2) a variety of cardiorespiratory events (e.g., bradycardia) leading to periods of decreased blood pressure; 3) respiratory problems associated with hypocarbia or hypoxemia and ventilator associated injury[37]. Preterm human periventricular cerebral white matter is supplied by the long vessels penetrating the cerebral wall from the pial surface[38]. At 24 to 28 weeks of gestation, these long penetrators have relatively few side branches and infrequent intraparenchymal anastomoses with each other[39]. These characteristics make this region an arterial end zone susceptible to ischemia in case of low perfusion. Also, the very low values of

volume flow (1.6-3.0 mL/100 g/min) in cerebral white matter in the human preterm infant and the absence of smooth muscle in the middle layer of penetrating cerebral arteries and arterioles in the third trimester in the human brain partially explain the propensity to impaired cerebral autoregulation and a pressure-passive circulation[40,41]. The abnormalities in cerebrovascular regulation are frequent in sick preterm infants[42]. In ventilated premature infants during the first hours of life, near-infrared spectroscopy (NIRS) demonstrated a pressure-passive cerebral circulation in more than half of premature infants[43]. Moreover, hypocarbic alkalosis can promote hypotension and indirectly increase the risk of white matter injury[44]. Indeed, the relationship between impaired cerebral blood flow and the occurrence of white matter injury is supported, in clinical practice, by the association of white matter injury with several markers of hypoxic-ischemic events: neonatal acidosis, hypovolemia, oliguria, abrupt decreases in blood pressure, patent ductus arteriosus, congenital heart disease or preterm infants submitted to cardiac surgery, infants treated with extracorporeal membrane oxygenation (ECMO) and infants with apneic spells and bradycardia[37].

Hypoxia can limit the survival of oligodendrocytes, a central cell affected in PVL. Hypoxia disrupts epidermal growth factor receptor-mediated mechanisms that are required for the survival and generation of new oligodendrocytes[45]. Furthermore, hypoxia promotes, via hypoxia-inducible factor, pre-oligodendrocytes (preOLs) maturation arrest through autocrine activation of the Wnt-beta-catenin pathway[46,47]. The preOLs are markedly more susceptible to hypoxia-ischemia and oxidative stress than other neural cell types[48,49]. In regions that sustained similar ischemic cerebral blood flow, the distribution of white matter injury corresponds to the distribution of preOLs[50].

Clinical presentation

In the preterm neonate, defining hypoxic–ischemic injury is more difficult than in the term neonate. Frequently, injury tends to occur in the context of risk factors, such as infection, inflammation, growth restriction, severe hypoglycemia, hypotension, hypoxia or hyperoxia, and only in some cases a sentinel event (e.g., cardiotocograph abnormalities with previously normal pattern, placental abruption, uterine rupture, cord prolapse) is recognized[51].

Preterm hypoxic–ischemic injury clinical course and outcomes remain incompletely elucidated. Studies that examine preterm HIE are heterogeneous, with variable inclusion criteria and outcomes reported. The incidence is probably higher than recognized and follows a more

complex clinical course, with higher rates of adverse neurological outcomes, compared to term infants[51]. Reported incidence across studies varies from 1.3 to 9/1000 live births[51].

Criteria used to identify preterm HIE are not yet well established and vary across studies, but include similar characteristics used in term infants. These comprise low Apgar scores at 1, 5 and 10 min, cord pH <7, base deficit >15 mmol/L, sentinel event, need for resuscitation and clinical evidence of encephalopathy (abnormal tone, posture, level of consciousness, spontaneous activity, primitive reflexes, and autonomic response) [52–54]. Preterm neonates with a delayed resolution of acidosis, renal impairment, raised creatinine, elevated liver enzymes, prolonged assisted ventilation, and abnormal neurological examination can be presumed to have suffered some degree of hypoxic insult[51].

MRI is the most sensitive imaging modality for the diagnosis of HIE in preterms, and focal non-cystic white matter injury is the most commonly recognized pattern of this kind of brain injury. Infants with white matter injury can also have basal ganglia and thalamic injury.

Outcome

Cerebral palsy or milder developmental coordination disorder, impairments in learning, cognition, memory, executive function, vision and hearing, as well as epilepsy and psychiatric disorders can all be, indeed, consequences of preterm birth[55].

There is strong evidence that PVL and intraventricular hemorrhage are prognostic factors for cerebral palsy in preterm children[56]. Spastic paresis is the common form of presentation of preterm children with major motor deficits and spastic paresis in preterms affects more the lower limbs than the upper limbs due to the topography of the focal lesions[37]. White matter cysts and/or signal abnormality on MRI located more anteriorly, near the corticospinal tracts, are most strongly associated with motor deficits[57]. Very preterm (VPT, 28-32 weeks) birth results in smaller basal ganglia and thalamic volumes at term-equivalent age, and these smaller volumes were related to a range of neurodevelopmental deficits, including motor deficits[58]. Of the VPT infants with motor impairment, 58% had complex MND and this dysfunction has been associated with poorer cognitive ability, and behavior[59,60]. In fact, up to 40% of VPT children are diagnosed with cognitive deficits at 2 years of age[61], have higher rates of language function problems compared with term children[62] and have a three-fold increased risk of psychiatric disorders in middle childhood[63]. The “preterm behavioral phenotype” include an increased risk for symptoms and disorders associated with inattention, anxiety, and social difficulties[63].

Attention-deficit/hyperactivity disorder is relatively common among preterm children, with prevalence rates of about 10%[64].

Some studies were specially designed to evaluate the outcome of preterm children with clinical criteria of HIE. Logitharajah et al showed that the two-year outcome in preterm HIE infants was death in 32%, cerebral palsy in 26% (mostly severe quadriplegia), mild impairment in 10%, and normal outcome in 32% of infants[52]. In another study, death or disability occurred in no infants with mild or moderate HIE, but in all infants with severe HIE[53]. A retrospective review of 1325 infants, 32–36 weeks gestational age, revealed an incidence of perinatal HIE of 0.9%, and a poor outcome was identified in 58%[54].

PERINATAL STROKE

Perinatal stroke is defined as an acute neurologic syndrome with chronic sequelae due to cerebral injury of vascular origin, occurring between 20 weeks of gestation and 28 days of postnatal life[65]. The major subtypes of perinatal stroke comprise perinatal arterial ischemic stroke, cerebral venous thrombosis and hemorrhagic stroke, which account, respectively, for 70, 20, and 10% of acute symptomatic perinatal stroke[66]. The incidence of perinatal arterial ischemic stroke has been estimated at 1 in 1600 to 5000 births[65,67]. Identification of a causative factor for perinatal arterial ischemic stroke remains difficult to achieve in most cases and there are no current means of prevention. A multifactorial etiology based on prenatal, perinatal and neonatal risk factors is presumed[68]. The hemorrhagic stroke can result from two main mechanisms: a primary hemorrhage resulting from vascular anomalies or bleeding diatheses; a secondary conversion of arterial or venous ischemic infarction[69].

Perinatal stroke is an important cause of chronic neurologic disability in children. In a previous study, Lee et al. described that after perinatal arterial ischemic stroke, 58% of infants developed motor deficits, 39% epilepsy, 25% language delay acquisition and 25% behavior problems[70].

ANIMAL MODELS OF NEONATAL HYPOXIA-ISCHEMIA

Important differences exist in brain development between human infants and rodent models. Gestation in humans lasts around 40 weeks and in rats lasts 23 days. From a developmental point of view, rodents are born with a more premature brain compared to humans. Despite the differences relative to the human species, the experimental models are crucial to the understanding of the pathophysiology and the development of potential therapeutics.

Hypoxia-ischemia has been extensively studied in rodents and to a lesser extent in larger animals such as pigs, sheep or primates[9]. There are several models of neonatal hypoxia-ischemia, but most published reports modelling neonatal hypoxia-ischemia in animals have employed the Rice–Vannucci model or variants[71]. The model comprises unilateral carotid artery ligation followed by exposure to 8% oxygen for 1–3 h at 37°C[71] and it replicates the human brain neonatal injury (thalamus, basal ganglia, cortex and hippocampus) and gives rise to well documented deficits, including impaired spatial learning and memory, reduced attention, impaired motor function and motor reflexes, and sensory processing abnormalities[9]. However, in this model, the lesion is unilateral (while in humans normally both hemispheres are affected), and there is some variability in the size and severity of the infarct due to the severity of the insult and collateral vessel compensation.

Other neonatal experimental models use a more moderated approach and focus on hypoxia without ischemia. Hypoxia is induced using an oxygen deprivation chamber. These models tend to provoke milder injuries, which could be advantageous if the aim is to mimic mild-moderate HIE and avoid the non-physiological occlusion of the carotid artery. The behavioral phenotype of this model includes hyperactivity, aggression, altered ultrasonic vocalization and altered sleep[9]. Chronic intermittent hypoxia simulates neonatal premature events, chronic continuous hypoxia mimics cyanotic congenital heart disease or chronic ventilatory impairment and acute hypoxia can be a model of ruptured uterus, placental abruption or difficult vaginal delivery[72].

In a different approach to hypoxia-ischemia models, the experimental intervention occurs before birth. Hypoxic or hypoxic-ischemic conditions are induced by either obstructing the blood supply to the uterus, asphyxiating the uterine horns, or placing the pregnant dam in hypoxic conditions. These models allow the study of more developmentally immature rodent brains. They have been suggested to mimic brain injuries associated with prematurity, fetal asphyxia, placental hypoperfusion, and intrauterine growth restriction. Uterine artery

occlusion is commonly performed on embryonic day 17 (E17) rats, which is thought to correspond to the early third trimester of human pregnancy. Ligation of the bilateral uterine arteries produces deficits that demonstrate a remarkable similarity with those found in humans with cerebral palsy[73]. The model causes disturbances in white matter and caudate nucleus volume and produces motor dysfunction. Transient occlusion of the uterine arteries in E18 for 60 minutes is proposed to be a clinically relevant model of the encephalopathy of prematurity and is associated with long-term functional deficits[74]. An alternative model to intrauterine hypoxic-ischemic injury involves excising the uterus horns and submerging them in water to induce anoxia. The model was developed to recapitulate the effects of acute asphyxia near birth[72].

STRATEGIES TO IMPROVE NEUROLOGIC OUTCOME IN ISCHEMIA

TH is the standard of care for moderate to severe neonatal HIE in infants ≥36 weeks gestation[75]. It is an effective therapy, though it is necessary to treat about 6 neonates with TH for one to be saved from death or severe disability at age 18–22 months[6]. Thus, TH is only partially effective and can only rescue some infants. Additional treatments are needed to improve the outcome of neonatal HIE. Currently, different therapeutic strategies include: 1) pharmacologic therapies (drugs with free radical scavenging, anti-apoptotic, anti-inflammatory, neuroregeneration, or vascular effects); 2) cell-based therapies (umbilical cord blood cells, placenta-derived stem cells, mesenchymal stem cells, and others); 3) microRNAs and 4) environmental enrichment therapies[76–79]. These distinctive approaches target the different phases of hypoxic-ischemia injury. High-effective therapies that target the primary phase of HIE would be perfect as they avoid the development of the subsequent phases and prevent secondary damage. Free radical scavenging and anti-excitotoxicity drugs could be good examples to reach this objective. Indeed, the selection of drugs that are already approved and used in clinical practice, is probably the fastest way to try to address the problem.

AIMS

The main objective of this thesis was to explore the neurologic consequences of hypoxic-ischemic injury in the neonatal brain using a translational etiologic approach. To achieve this, the study specific objectives include:

- Characterization of the clinical presentation and outcome of PVL in preterm children.
- Characterization of the clinical presentation and outcome of perinatal stroke in term children.
- Assessment of the neurologic consequences of neonatal HIE, beyond cerebral palsy, in the term children.
- Assessment of the neurodevelopmental and behavioural consequences of prematurity using non-inflammatory and inflammatory rat models of preterm brain injury.
- Investigation, in vitro, of potential new treatments to ameliorate hypoxia-ischemia lesions.

These objectives were achieved by carrying out a series of studies, the results of which are reported in the following works:

1) **"Neurodevelopmental outcomes of children with periventricular leukomalacia: the role of infection and ischemia"** (Leite et al., 2021). The goal of this study was to assess the neurodevelopmental outcomes of children with PVL and its etiologic subgroups. This was a retrospective review of medical records of children with PVL, born at a tertiary center between 1996 and 2016. The sample was divided into two groups according to the most likely etiology of PVL, using a classification system of risk factors. The neurologic and development outcomes were reviewed.

2) **Ischemic and hemorrhagic perinatal stroke in a neonatal intensive care unit: a 10-years survey"** (Fonseca et al., 2018). In this retrospective study we sought to study the risk factors, clinical manifestations and follow-up of newborns admitted to a tertiary center between

January 2006 and December 2015 with the final diagnosis of perinatal stroke. The selected perinatal stroke cases were those with MRI confirmation and the selected risk factors to be examined were chosen based on recent review studies or meta-analyses.

3) **"Neonatal hypoxic-ischaemic encephalopathy: motor impairment beyond cerebral palsy"** (Erdi-Krausz and Rocha et al., 2021). We investigated school-age neurologic and neuromotor functions of children with HIE without cerebral palsy and correlate them with attention and general cognitive function, neuromotor assessments at toddler age and neonatal MRI. Twenty-seven children with neonatal HIE without cerebral palsy who underwent TH and a comparison group of 20 children were assessed at age 5–7 years for MND, motor skills, parental concern over motor function, general cognition and attention. Neurologic examination and motor development, using Bayley-3 Scales, at age 24-months was extracted from a clinical database. Neonatal brain MRI was assessed for hypoxic-ischemic injury.

4) **"Behavioral and brain morphological analysis of non-inflammatory and inflammatory rat models of preterm brain injury"** (Rocha et al., 2021). We intended to evaluate whether the neurodevelopmental and behavioral consequences of preterm birth, induced by a non-inflammatory model of preterm birth using mifepristone, would differ from those induced by an inflammatory prenatal transient hypoxia-ischemia (TSHI) model. Rat pups were tested postnatally for the characterization of developmental milestones and behavioral and cognitive functions, namely anxiety, spatial learning and memory. Brains were processed for quantification of doublecortin- and neuropeptide Y-immunoreactive cells, and cholinergic varicosities in the hippocampus.

5) **"Repurposed edaravone, metformin, and perampanel as a potential treatment for hypoxia–ischemia encephalopathy: an in vitro study"** (Silva et al., 2022). In this study, we wanted to test three repurposed drugs (edaravone, perampanel, and metformin) to evaluate if they could revert, or at least attenuate, the deleterious effects of hypoxia–ischemia in an in vitro model. We analysed two different cell lines (HT-22 and SH-SY5Y) through cell viability assays, morphology analysis, and detection of reactive oxygen species production.

PUBLICATIONS

NEURODEVELOPMENTAL OUTCOMES OF CHILDREN WITH PERIVENTRICULAR LEUKOMALACIA: THE ROLE OF INFECTION AND ISCHEMIA

Leite SS, Matos J, Grenha J, Braga AC, Rocha R.
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In this work, the candidate was responsible for the design and methodology of the study, participated in the description and statistical analysis of the results, contributed to their interpretation and discussion and did the critical review of the manuscript.

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Original article

Neurodevelopmental outcomes of children with periventricular leukomalacia: the role of infection and ischemia

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Abstract

Introduction: Periventricular leukomalacia (PVL) is an important cause of preterm newborn's cerebral white matter disease. This study assessed neurodevelopmental outcomes of children with PVL and its etiologic subgroups.

Methods: Retrospective review of medical records of children with PVL diagnosis born at a tertiary center between 1996 and 2016. Subjects were divided into two groups according to the most likely etiology of PVL (ischemic versus infectious) using a classification system of risk factors. The neurologic and development outcomes were reviewed.

Results: A total of 34 newborns with a median gestational age of 29 weeks were selected. Sixteen newborns (51.6%) were included in the ischemic group, while 15 (48.4%) were included in the infectious group; a clear group classification was not possible in 3 cases. PVL was moderate to severe in 73.5% of cases. Cerebral palsy (CP) developed in 69.7% of the children, 29% had epilepsy and 15.6% were microcephalic. Children with moderate to severe PVL were significantly more impaired than children with mild PVL ($p < 0.05$). Moderate to severe PVL was observed in 93.3% of the children in the infectious group and 71.4% in the ischemic group ($p = 0.12$). Children in the infectious group were more prone to abnormal development and CP, while children in the ischemic group had more epilepsy and hearing impairment than the infectious group.

Discussion: Infection may be an important etiologic factor regarding severe forms of PVL. The infectious group presented a higher incidence of CP, which may be related to more severe white matter injuries. The ischemic group presented more epilepsy, suggesting the involvement of gray matter disease.

Keywords

Periventricular leukomalacia, etiology, cerebral palsy, neonatal brain white matter injury, cognitive outcome.

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Introduction

Significant advances in neonatal assistance have improved the survival of extreme preterm neonates. However, neurodevelopmental sequelae, such as cerebral palsy (CP), cognitive impairment and behavioral changes, remain a major concern [1].

In a developing organism, any insult to the central nervous system may have permanent consequences, depending not only on the nature of the insult but also on the timing in which it occurs. The brain, particularly the preterm's cerebral white matter, is extremely susceptible to ischemic and infectious insults. The two main brain lesions responsible for neurological sequelae in preterm newborns are periventricular leukomalacia (PVL) and periventricular hemorrhagic stroke [2].

PVL is the leading cause of non-hemorrhagic neuropathological abnormality in the cerebral white matter of a preterm infant [3].

From the anatomopathological point of view, PVL has two basic components: a deeper zone of focal necrosis and a more peripheral and extensive area where diffuse gliosis predominates. Histologically it is characterized by coagulation necrosis, microglial infiltration, astrocytic proliferation, and eventual cyst formation. The ultimate result is the loss of volume of cerebral white matter with secondary ventriculomegaly [4].

Previous data indicate that the pathogenesis of PVL is the consequence of the effect of hypoxia, ischemia and inflammation in the progenitor oligodendrocyte cells, which are particularly vulnerable to such insults, and are located in the periventricular area between the 23rd and 32nd weeks of gestation [5].

The main factors that explain PVL anatomic distribution and higher incidence in preterms are the distribution and development of brain vascularization, the lack of cerebral blood flow autoregulation and the intrinsic vulnerability of the cerebral white matter, rich in pre-oligodendrocytes. Any inflammatory or infectious process leads to systemic upregulation of pro-inflammatory cytokines and diffuse activation of microglia within the immature white matter, leading to injury [6].

The aim of this study was to analyze the neurodevelopmental outcomes of children with PVL. Additionally, we assessed the neurodevelopmental outcomes according to two major insults in PVL (ischemic and infectious), so as to help clarify the weight of each insult in the prognosis of PVL.

Methods

This study was conducted at a tertiary-care hospital. Medical records of preterm babies, born between 1996 and 2016, diagnosed with PVL, were retrospectively reviewed. The diagnosis was established by ultrasound, performed by expert neonatologists. Data concerning demographic and clinical characteristics were obtained from clinical records.

Based on brain ultrasound findings, PVL was classified in groups using a modified version of the classic classification: grade 1, transient periventricular echo densities persisting for > 7 days; grade 2, transient periventricular echo densities evolving into small, localized frontoparietal cysts; grade 3, periventricular echo densities evolving into extensive periventricular cystic lesions; grade 4, densities extending into the deep white matter evolving into extensive cystic lesions [7]. Grade 1 was classified as mild PVL and grade 2 to 4 as moderate/severe PVL.

All cases were classified by two independent researchers, according to the prenatal and neonatal history, in one of two groups: infectious group and ischemic group. The classification was carried out by scoring one point to the following risk factors: for presumed infectious origin – prolonged rupture of membranes (> 12 h), chorioamnionitis, maternal

fever, maternal leucocytosis, a high level of C-reactive protein (CRP), infectious pathological changes in the placenta and neonatal sepsis; for presumed ischemic origin – intrauterine growth restriction, preeclampsia, umbilical cord knot, placental abruption, ischemic pathological changes in the placenta, multiple pregnancies, hypoxemia and apneas with respiratory support, anaemia, hypotension and patent ductus arteriosus. The highest score in each cluster of risk factors defined the group.

Chorioamnionitis was established in the presence of fever of 37.8ºC or higher plus two or more minor signs (maternal tachycardia > 100 beats/min, fetal tachycardia > 160 beats/min, uterine tenderness, foul odour of the amniotic fluid and leucocytosis > 15,000/mm^3) [8]. Significant hypotension was considered in cases that needed resuscitation with fluids or drugs.

All children were regularly followed as out-patients upon hospital discharge and they all had at least two years of follow-up. The evaluation was performed using the Griffiths Developmental Scale. The results were presented according to the common metring system [9]. Audiology assessment through the auditory brainstem response was performed at least until 24 months of age. The children were also observed by an ophthalmologist during their stay at the Neonatal Intesive Care Unit (NICU) to identify signs of retinopathy and after discharge in the clinic.

CP diagnosis was performed by the neonatologist responsible for the follow-up of high-risk preterm babies. CP was classified as spastic, ataxic, and dyskinetic [10].

Data analysis was performed using the Statistical Package for the Social Sciences for Windows® (SPSS® version 25.0, IBM® SPSS Incorporated, Chicago, IL). Univariable analysis was performed through Chi-square tests (for categorical variables), t-tests (for categorical versus continuous variables with normal distribution) and U-Mann-Whitney tests (for categorical versus continuous variables without normal distribution). Multivariable analysis was not feasible due to the reduced number of patients included. A p-value of less than 0.05 was considered statistically significant.

According to the Institutional Review Board of our institution, all data was anonymised for record and subsequent analysis.

Results

A total of 34 newborns (55.9% male) with a median (P25-P75) gestational age of 29 (28-30) weeks were selected. From these, collected data allowed for group classification in 31 of the newborns: 16 (51.6%) mainly had ischemic risk factors thus classified in the ischemic group and 15 (48.4%) predominantly had infectious risk factors thus classified in the infectious group. Three newborns were excluded from the group classification because they had a mixture of ischemic and infectious risk factors, which did not enable a clear classification.

Demographic data, prenatal history and delivery details are described in **Tab. 1**.

Prolonged rupture of membranes occurred in 12 (37.5%) newborns, while chorioamnionitis occurred in 7 (21.9%). Assisted vaginal delivery or C-section occurred in 58.8% of the cases. There were 4 cases of placental abruption and 1 of knot of the umbilical cord.

Placental pathology report available for 18 newborns had ischemic findings in 5.6% of cases, infectious findings in 61.1% and a mixture of alterations in 5.6%. Placental pathology identified ischemia in 10.0% (1/10) of the newborns in the ischemic etiology group and inflammatory changes in 72.7% (8/11) of those in the infectious group.

The median length of total stay was 47 (33-63) days. PVL was classified as mild in 6 cases, moderate to severe in 25, while in 3 cases it was not possible to grade. Median days for PVL diagnosis were 6 (2.7-13.2), 53.8% being diagnosed during the first week of life. During hospitalization 87.5% of newborns needed respiratory support: invasive in 62.5% (n = 20) and only non-invasive in 43.8% (n = 14). Invasive ventilation needs were significantly more frequent in newborns with moderate to severe PVL when compared with mild PVL (n = 17, $p < 0.05$). Early neonatal sepsis occurred in 38.2%. Despite not statistically significant ($p = 0.057$), newborns with early-onset sepsis developed only moderate to severe PVL and not mild PVL. Intraventricular hemorrhage was identified in 25%. Retinopathy was present in 6 newborns (grade 1 in 5 cases).

All children were submitted to neurodevelopmental assessments for a minimum of 2 years. Nine children (26.5%) had a normal developmental outcome and the remainder (73.5%) had different grades of impairment in different areas (**Tab. 2**).

CP developed in 69.7% of the children, the majority of whom had spastic CP (63.6%). Microcephaly was present in 15.6% of the children and 29% had epilepsy. Every child who developed epilepsy belonged to the moderate to severe PVL group.

Table 1. Demographic data and hospitalization details.

Variables (n = 34 [a])		n (%)
Sex (male)		19 (55.9%)
Weight at birth (mean, min-max), g		1,305 (720-1,960)
Gestational age (median, P25-P75), weeks		29 (28-30)
Prenatal history		
Number of fetuses	1	21 (61.8%)
	2	12 (35.3%)
	3	1 (2.9%)
Intrauterine growth restriction		3 (8.8%)
Preeclampsia		2 (5.9%)
Prenatal steroids		25 (73.5%)
Delivery details		
Vaginal delivery		14 (41.2%)
Assisted vaginal delivery or C-section		20 (58.8%)
Placental abruption (n = 33)		4 (12.1%)
Chorioamnionitis (n = 32)		7 (21.9%)
Placental pathology (n = 18)	Ischemic findings	1 (5.6%)
	Infection findings	11 (61.1%)
	Overlapping findings	1 (5.6%)
	Feto-fetal transfusion syndrome	2 (11.1%)
	Non-specific	3 (16.7%)
Hospitalization details		
Length of stay (median, P25-P75), days		47 (33-63)
Need of respiratory support (n = 32)		28 (87.5%)
Non-invasive ventilation		14 (43.8%)
Invasive ventilation		20 (62.5%)
Anemia with need of transfusion (n = 32)		8 (25%)
Hypotension (n = 32)		9 (28.1%)
Arterial duct patency (n = 32)		10 (31.3%)
Neonatal sepsis (n = 32)		23 (71.9%)
Retinopathy (n = 33)		6 (18.2%)
Grade 1		5 (15.2%)
Grade 2		1 (3%)
Intraventricular hemorrhage (n = 32)		8 (25%)
PVL	Grade 1	6 (17.6%)
	Grade 2	10 (29.4%)
	Grade 3	14 (41.2%)
	Grade 4	1 (3%)
	Non-classified	3 (8.8%)

[a] Unless otherwise specified.
PVL: periventricular leukomalacia.

Table 2. Follow-up data of patients with periventricular leukomalacia (PVL).

Outcome variables (n = 34 [a])		n (%)
Normal development		9 (26.5%)
Normal hearing (n = 32)		29 (90.6%)
Ophtalmologic evaluation (n = 32)	Normal	15 (46.9%)
	Refractive errors	3 (9.4%)
	Strabismus	12 (37.5%)
	Visual impairment	2 (6.3%)
CP (n = 33)		23 (69.7%)
Spastic		21 (63.6%)
Dyskinetic		5 (15.2%)
Epilepsy (n = 31)		9 (29%)
Microcephaly (n = 32)		5 (15.6%)

[a] Unless otherwise specified.
CP: cerebral palsy.

During follow-up, 9.4% were found to have hearing loss and 53.1% altered ophthalmic evaluations (**Tab. 2**).

To assess quantitative development, Griffiths mental development scale was used in children who were approximately 3 years old. Only about a third (n = 10) of the patients were evaluated by Griffiths scale, either because of severe neurodevelopment limitations or lack of cooperation. Results were below average with greater expression in the locomotor development subscale (QA), especially in moderate to severe PVL. Children with moderate to severe PVL had significantly more neurodevelopmental complications than children with mild PVL during follow-up ($p < 0.05$). **Fig. 1** presents Griffiths subscales results according to PVL grade. **Fig. 2** plots the results of subscales regarding the two groups.

When we compared the ischemic and infectious groups (**Tab. 3**), moderate to severe PVL was seen in 93.3% of the children in the inflammatory group, and in 71.4% of the ischemic group ($p = 0.12$).

Children in the infectious group were more prone to abnormal development (80% vs. 62.5%, $p = 0.28$). CP also occurred more in the infectious group compared with the ischemic group (85.7% vs. 56.3%, $p = 0.11$). Children in the ischemic group had more epilepsy and hearing impairment than the infectious group.

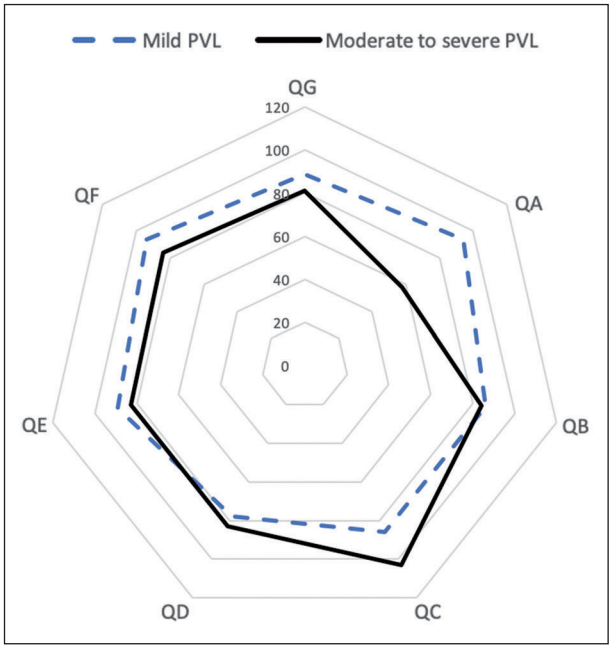

Figure 1. Relation between Griffiths Mental Development Subcales (median scores) and periventricular leukomalacia (PVL) groups.
QG: total scale; QA: locomotor development; QB: personal-social development; QC: hearing and speech; QD: hand and eye coordination; QE: performance tests; QF: practical reasoning.

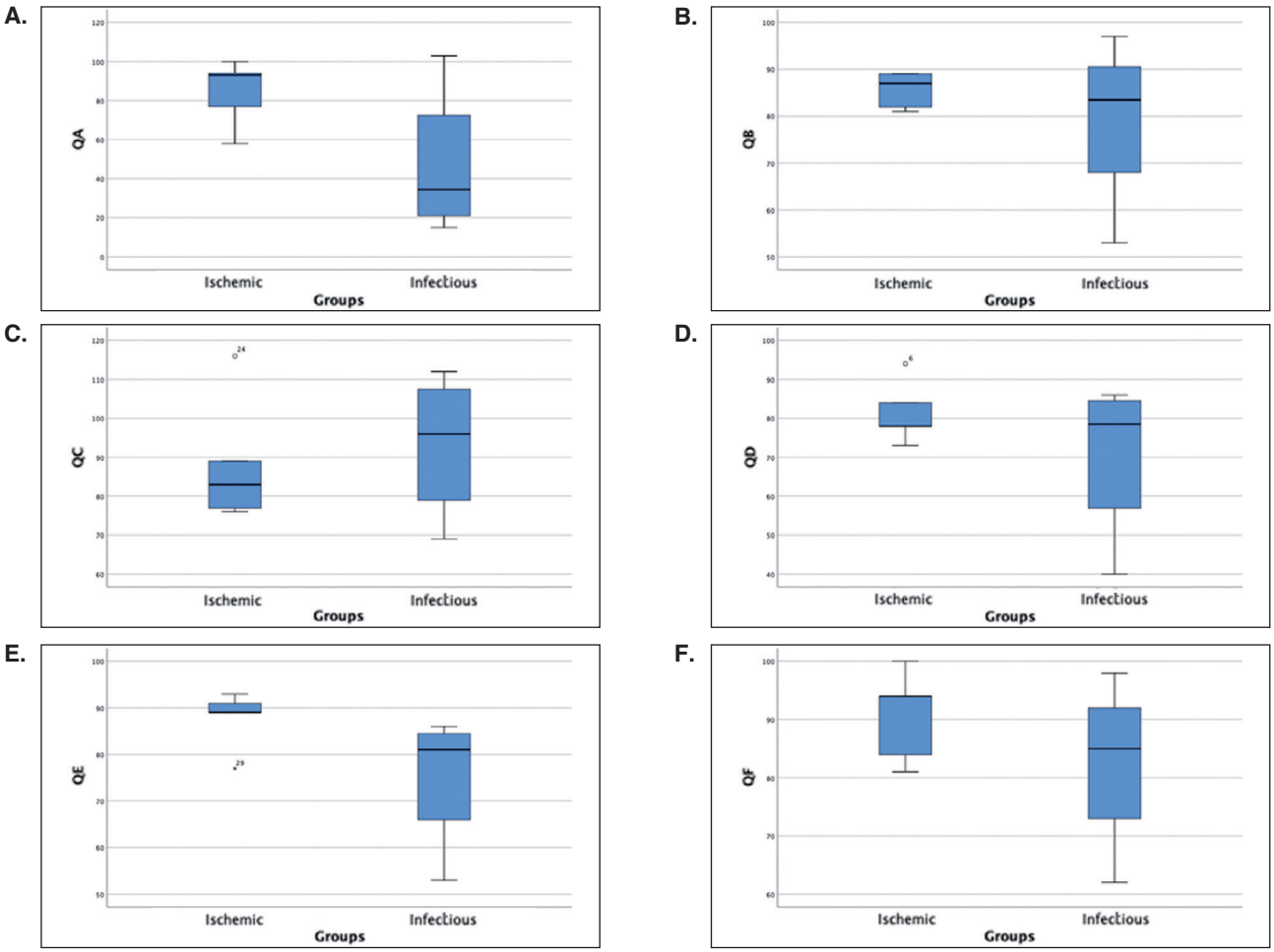

Figure 2. Relation between Griffiths Mental Development Scales (median scores) and etiologic groups.
QA: locomotor development; QB: personal-social development; QC: hearing and speech; QD: hand and eye coordination; QE: performance tests; QF: practical reasoning.

Table 3. Results of univariate analysis ischemic vs. infectious etiology group.

		Ischemic group (n = 16)	Infectious group (n = 15)	p
Sex (male)		8 (50%)	9 (60%)	0.72
Weight at birth (mean, SD), g		1,159 ± 206	1,421 ± 251	0.01
Gestational age (median), weeks		29 ± 1.8	30 ± 1.8	0.08
Chorioamnionitis		1/15 (6.7%)	6/13 (46.2%)	0.02
Placental pathology	**Infection findings**	3/10 (30%)	8/11 (72.7%)	0.31
	Ischemic findings	1/10 (10%)	0 (0%)	
	Overlap findings	1/10 (10%)	0 (0%)	
	Unspecific findings	3/10 (30%)	2/11 (18.2%)	
	Normal	2/10 (20%)	1/11 (9.1%)	
PVL	**Grade 1**	4/14 (28.6%)	1/15 (6.7%)	0.12
	Grade 2	6/14 (42.9%)	4/15 (26.7%)	
	Grade 3	4/14 (28.6%)	10/15 (66.7%)	
Intraventricular hemorrhage	**Grade 1**	1/16 (6.3%)	0/15 (0%)	0.13
	Grade 2	1/16 (6.3%)	1/15 (6.7%)	
	Grade 3	4/16 (25%)	0/15 (0%)	
Normal development		6/16 (37.5%)	3/15 (20%)	0.28
CP		9/16 (56.3%)	12/14 (85.7%)	0.11
Spastic unilateral		3/9 (33.3%)	0/12 (0%)	
Spastic bilateral		5/9 (55.6%)	11/12 (91.7%)	
Dyskinetic		1/9 (11.1%)	1/12 (8.3%)	
Epilepsy		5/16 (31.3%)	3/13 (23.1%)	0.28
Microcephaly		3/16 (18.7%)	1/14 (7.1%)	0.37
Retinopathy		4/16 (25%)	2/15 (13.3%)	0.54
Hearing impairment		3/16 (18.7%)	0/14 (0%)	0.22
Visual impairment		1/16 (6.3%)	1/14 (7.1%)	0.90

CP: cerebral palsy; PVL: periventricular leukomalacia.

Discussion

PVL has originally been attributed to ischemic insults; however, more recent studies suggest that infectious and inflammatory diseases are also important in the pathological, histologic, and structural changes that characterize PVL [11].

This study has assessed the outcome of patients with PVL who were admitted to our institution throughout a decade. Our overall findings were in accordance with previous series [12-14]. We only found 6 cases of mild PVL (grade 1). A possible explanation can be the failure to register mild cases in the hospital database. Another explanation can be the fact that cerebral ultrasound is less sensitive to minor and diffused white matter lesions, which can lead to underdiagnosis. However, cerebral ultrasonography is still the most widely used imaging technique in NICU, as it is a quick, non-invasive test that can be performed at the patient's bedside, making it the ideal screening tool for PVL. It is very important to perform sequential evaluations, especially in the first week of life, and continue this screening until the term age so as to diagnose the majority of cases of PVL [15]. Most newborns with PVL do not show clinical manifestations in early life; therefore, mild PVL may go by unnoticed. Magnetic resonance imaging (MRI) is a more accurate mean for PVL diagnosis; nonetheless, it is more expensive and less accessible, making its use limited in daily clinical practice [16, 17].

We found more cases of moderate to severe PVL in the infectious group as well as a higher prevalence of placental changes suggestive of infection. This suggests that infection may cause more prominent histologic and structural abnormalities in white matter than ischemia, which may also lead to a more serious form of PVL. It is established that the inflammatory pathway, mediated by cytokines, is highly involved in the nervous cell death by neuronal apoptosis [18]. The presence of infection may function as a coadjuvant, increasing vulnerability for minor ischemic insults, which alone would not be sufficient to cause injury. In a study with 172 newborns, Yoon and collaborators demonstrated that umbilical cord plasma IL-6 concentration was a significant predictor of PVL [19]. Leviton et al. published for the first time in 1976 that neonatal sepsis was related to PVL [20]. Moreover, there is strong evidence that very low birth weight infants with neonatal sepsis have a higher risk for white matter lesions and CP [21]. Accordingly, chorioamnionitis is a known risk factor for both CP and cystic PVL [22]. In our study, newborns with early-onset sepsis also had more severe PVL. These data support the role of infection in increasing the severity of PVL, which reinforces the importance of an adequate prenatal and postnatal infection management.

In our placental pathology reports, ischemic lesions were described in only 1 case against 11 cases with descriptions suggestive of infection. This could also reinforce the assumption that inflammation early in life (in the uterus) is probably an important contributor to white matter injury. Pathologic examination of the placenta should be routinely performed in all preterm deliveries to identify aetiologies that can predict different outcomes.

Concerning neurodevelopmental outcomes, as expected, impairments were more prevalent in the moderate to severe PVL group. The infectious group presented more abnormal development trajectories and a higher incidence of CP than the ischemic aetiology group, reflecting a more severe white matter injury.

In the neurodevelopmental assessment by Griffiths scale, children showed results below average, with greater expression in the QA. QA was considerably lower in the moderate to severe PVL group, a fact that has been described in the literature and correlates with the anatomic distribution of PVL [14]. QA was also lower in the infectious group compared to the ischemic group, which reflects the presence of more severe lesions in the former. Among the children submitted to the Griffiths scale (n = 10), 6 children (60%) had mild development delay and just 1 (10%) presented severe delay. This is related to the fact that the most severe cases were not evaluated through the Griffiths scale.

We found evidence of increased ophthalmologic complications in the moderate to severe PVL group, with strabismus being the most frequent finding, thus concordant with previous literature [23, 24].

Limitations

This study had some limitations: the limited number of patients in the mild PVL group, the difficulty to obtain complete data from newborns born between 1996 and 2016 (especially previous to computerized records) inherent to a retrospective study and also the small size of the sample, which did not allow for a wider validation through multivariate analysis. Additionally, neonatal brain MRI would have been useful for a better characterization of PVL disease and correlation with outcomes, especially in mild PVL.

Conclusions

Despite the limitations, to the best of our knowledge, this was the first study that has tried to identify a relationship between the type of insult (ischemic vs. infectious) based on risk factors and neurological outcomes of the preterm newborn. Infection seems to be a stronger trigger for PVL, so every effort should be made to prevent infection in preterm and this should be regarded as an additional neuroprotection measure to be aware of in the NICUs.

Preterm babies with PVL are at risk of several complications and neurodevelopment disorders. Further studies should be performed to identify patient subgroups with different outcomes that may benefit from specific types of interventions.

Our work pointed out that the infectious group presented a higher incidence of CP, which may be related to more severe white matter injury. In the ischemic group, epilepsy was more prevalent, suggesting the involvement of gray matter disease.

Declaration of interest

The Authors declare that there is no conflict of interest.

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ISCHEMIC AND HEMORRHAGIC PERINATAL STROKE IN A NEONATAL INTENSIVE CARE UNIT: A 10-YEARS SURVEY

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Ischemic and Hemorrhagic Perinatal Stroke in a Neonatal Intensive Care Unit: A 10-Years Survey

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Abstract

***Background:** Perinatal stroke has been associated with a multifactorial etiology and it is an important cause of long term neurologic impairment.*

***Methods:** This is a retrospective study on risk factors, clinical manifestations and follow-up of newborns admitted in a level three Neonatal Intensive Care Unit between January 2006 and December 2015 with the final diagnosis of perinatal stroke. The selected perinatal stroke cases were those with magnetic resonance imaging confirmation and the selected risk factors were chosen based on recent review studies or meta-analyzes.*

***Results:** Seventeen cases were identified (13 ischemic and four hemorrhagic) and the most common clinical presentation was seizure, 20 to 48 hours after delivery, occurring in 88% of newborns. All ischemic stroke cases had at least one specific risk factor, and 60% had more than three. Only one hemorrhagic stroke case had a specific risk factor. Four placentas had histological abnormalities. A prothrombin gene mutation was detected in one ischemic stroke case. There was no stroke recurrence or death, with a mean follow-up of 43 months. 62% of the infants had neurologic sequelae (motor impairment and epilepsy). All ischemic stroke cases with more than two risk factors had neurologic sequelae.*

***Conclusions:** Ischemic stroke cases usually presented specific risk factors. An association between risk factors´ number and a worse neurologic outcome was also observed in ischemic stroke cases.*

Keywords: Perinatal stroke, neonatal seizures, newborn, risk factors

Introduction

Perinatal stroke (PS) is currently defined as an acute neurologic syndrome with chronic sequelae due to cerebral injury of vascular origin, occurring between 20 weeks of gestation and 28 days of postnatal life.[1] Perinatal arterial ischemic stroke (AIS) has an estimated incidence of one in 2300 to one in 5000 newborns (NB) which exceeds 10 times the childhood stroke.[1,2] Its incidence ranks only second to stroke in the general population (adult and pediatric stroke).[1] The major subtypes comprise AIS, cerebral venous thrombosis and hemorrhagic stroke (HS) which account respectively for 70, 20, and 10 percent (%) of acute symptomatic PS.[2] Temporal classification based on neuroimaging and clinical features comprises fetal, neonatal and perinatal presumed stroke.[2-4] A multifactorial etiology based on prenatal, perinatal and neonatal risk factors is presumed.[4]

Identification of a causative factor for PS remains difficult to achieve in most cases with no current means of prevention.[5]

Pathophysiology of AIS includes thromboembolism, vasculopathy of cerebral arteries and hemostatic disturbances, but globally is poorly understood with most cases remaining idiopathic. The HS can result from two main mechanisms: a primary hemorrhage resulting from vascular anomalies or bleeding diatheses; a secondary conversion of arterial or venous ischemic infarction.[6]

Prothrombotic disorders have been associated with AIS but substantial levels of evidence are limited.[7-10] Current studies argue that rates of thrombophilia in children with PS are similar to those in the normal population and routine testing for thrombophilia should not be done.[7,10] Current guidelines recommend anticoagulation treatment only for NB with proven cardioembolic stroke.[11changed]

Perinatal stroke is an important cause of chronic neurologic disability in children and some authors state that perinatal AIS is the most common identifiable cause of cerebral palsy.[6] In a previous study, Béjot et al. described that after PS, 39% infants will develop epilepsy, 58% motor deficits, 25% language delay acquisition and 25% behavior problems.[7] Even in infants with an early reassuring development, long term neurodevelopmental follow-up should be accomplished.[12]

The aim of this study was a comparison between AIS and HS in a PS case series, characterizing clinical data as well as laboratory and imaging features; identifying already recognized risk factors for each subtype and evaluating neurologic outcome. Comparing neurologic outcome based on the number of risk factors was also targeted.

Material and Methods

Participant Selection

Subjects were identified from a Neonatal Intensive Care Unit integrated in a perinatal center with inborn babies of an Oporto single tertiary stand-alone children's hospital. Registries were recorded from the electronic medical database of all babies born from 2006 and 2015 with final diagnosis of stroke, confirmed by magnetic resonance imaging (MRI).

Exclusion criteria were outborn NB, preterm intraperiventricular hemorrhage associated with venous infarcts, periventricular leukomalacia, hypoxic-ischemic encephalopathy and traumatic lesions. Neuroimaging studies of all neonates were reviewed by experienced pediatric neurologists and neuroradiologists.

Data Acquisition

Clinical data were obtained by medical records. They included mode of delivery, Apgar scores, need for resuscitation, gestational age, birth weight, sex, age at presentation, clinical manifestation, age of diagnosis by MRI and hospitalization length. Laboratory and neurophysiological data were also collected. Cerebral ultrasound (US) and MRI studies were performed to determine the stroke characteristics, affected vessels, and location of the lesions.

The risk factors considered in this study for both AIS and HS were based on a recent meta-analysis on AIS risk factors´ and recent publications related to HS, respectively.[4,9,12,13] The risk factors for AIS considered in this study included: oligohydramnios, pre-eclampsia, intrapartum fever >38ºC, abnormal cardiotocography (CTG), meconium-stained amniotic fluid, cord abnormalities, instrument-assisted (forceps or vacuum) delivery, emergency cesarean section (CS), umbilical arterial pH <7.10, male gender, small for gestational age (SGA), need for resuscitation at birth, Apgar score of less than seven at five minutes and hypoglycemia (blood glucose 2.6 mmol/L) within the first two days after birth. The risk factors for HS considered were: fetal distress, placental abruption, complex congenital heart disease, neonatal sepsis, hemostatic abnormalities (low platelet count, prolonged prothrombin time, partial thromboplastin time and activated clotting time, low fibrinogen, congenital bleeding disorder and heparin exposure) and postmaturity (babies born after 42 weeks).[14]

Abnormal CTG included persistent late or variable decelerations, fetal bradycardia and/or reduced fetal heart variability.[4] Fetal distress corresponded only to a non-reassuring fetal heart tracing and/or decreased fetal movement prior to delivery.[12] Considered cord abnormalities were cord entanglements, hypercoiling, true knots, strictures and short cords.[4] Resuscitation at birth included the need of intubation for ventilation with or without cardiac compressions and/or epinephrine.

Other unclear or less described risk factors for PS as abnormal prothrombotic studies and placenta abnormalities were separately recorded.[3,6]

Outcome data were obtained from medical records of follow-up medical appointments and included death, stroke recurrence, post-stroke anti-epileptic drug treatment (at sixth and eighteenth month), neurologic sequelae and neurodevelopmental outcome (with in a minimum age of 18 months). Abnormal neurologic outcome was defined by the presence of hemiparesis and/or epilepsy. Speech problems were searched in children from the age 24 months. Neurodevelopmental global assessment was obtained by Griffiths Mental

Development Scale or clinical evaluation by a developmental pediatrician.

Data Analysis

Data were analyzed using SPSS statistical software, version 22.0 (SPSS Inc., Chicago, IL, USA). Categorical variables were summarized using frequencies (n) and percentages (%). For continuous variables, data were summarized by the median with interquartile range (IQR).

Results

Subjects And Clinical Manifestations

Out of 31826 live births between January 2006 and December 2015, there were 17 (0.0005%) PS cases identified, 13 AIS and 4 HS. Male represented 76% and 24% were preterm NB. Detailed demographic and clinical data for each stroke subtype are listed in Table 1.

Table 1. *Demographic, clinical and neuroimaging/neurophysiologic data.*

Data/ Stroke subtype	Ischemic (n=13) n (%) or median (IQR)	Hemorrhagic (n=4) n (%) or median (IQR)	Total (n=17) n (%) or median (IQR)
Demographic and clinical data			
Male gender	9 (69)	4 (100)	13 (76)
GA (weeks)	38 (36-39)	39 (36-39)	39 (36-39)
GA vs. BW (g)	3135 (2700-3530)	2970 (2745-3515)	2970 (2745-3515)
AGA	12 (92)	4 (100)	16 (94)
LGA	1(8)	-	1(6)
SGA	-	-	-
Newborn twins	2 (15)	-	2(12)
Eutocic delivery	4 (31)	3 (75)	7 (41)
Vacuum delivery	2 (15)	1 (25)	3 (18)
Forceps delivery	1 (8)	-	1 (6)
Cesarean section	6 (46)	-	6 (35)
1-min Apgar score	8 (5-9)	8 (5-9)	8 (5-9)
5-min Apgar score	10 (8-10)	10 (8.5-10)	10 (8.5-10)
Hospitalization (days)	14 (7-15)	13 (7-15)	13 (7-15)
Clinical manifestations			
Seizure	11 (85)	4 (100)	15 (88)
Time of presentation (h)	24 (20-48)	37 (24-48)	24 (24-48)
Hemisphere localizing clinical seizures	8 (62)	3 (75)	11 (65)
Feeding problems	5 (38)	1 (25)	6 (35)
Apnoeia	2 (15)	-	2 (12)
Cyanosis	2 (15)	-	2 (12)
Altered limb tone	1 (8)	-	1 (6)
Neuroimaging and neurophysiologic studies			
Cranial US:			
Normal	3 (23)	2 (50)	5 (29)

Abnormal	10 (77)	2 (50)	12 (70)
Diagnosis by MRI (day)	8 (6-12)	8 (6-12.5)	8 (6-12.5)
Brain affected territory (MRI)			
Left MCA	10 (77)	n.a.	10 (59)
Right MCA	1 (8)	n.a.	1 (6)
Watershed	2 (15)	n.a.	2 (12)
EEG†			
Normal	1 (8)	2 (50)	3 (18)
Abnormal	11 (92)	2 (50)	13 (76)

AGA = Adequate for gestational age, BW = Birth weight, EEG = Electroencephalography, GA = Gestational age, h = hours, LGA = Large for gestational age, min = minute, n.a. = not applicable, SGA = Small for gestational age, † One asymptomatic case did not receive EEG evaluation

In this cohort, all symptomatic cases included NB which developed seizures, being a total of 15 NB (88% of all PS cases). The median onset time of symptoms was 24 hours for AIS and 37 hours for HS and hemisphere localizing clinical seizures were found in 65% of cases. The two asymptomatic cases (AIS cases) were born at 36 weeks of gestation: one case had a history of fetal distress and emergent cesarean; the other case had a history of placental abruption and a vacuum delivery. Both stroke cases were suspected by neonatal cerebral US.

Imaging And Neurophysiological Studies

Cerebral US was performed in all NB included in this study (70% abnormal). Median age for diagnosis confirmation by MRI was the eighth day for both stroke groups. The two cases without clinical seizures were diagnosed later at ninth and thirteenth day by MRI, after a suspicious neonatal cranial US. Most frequently affected cerebral territory in MRI was that of left middle cerebral artery (MCA) (59%). Amplitude-integrated electroencephalography (aEEG) was performed in five neonates and was abnormal in the three AIS cases and normal in 2 the HS cases. Electroencephalography (EEG) was done in 16 cases (81% abnormal) – main results and distribution patterns are summarized in Table 1. Illustrative case images of AIS subtype can be seen on Figure 1 and Figure 2.

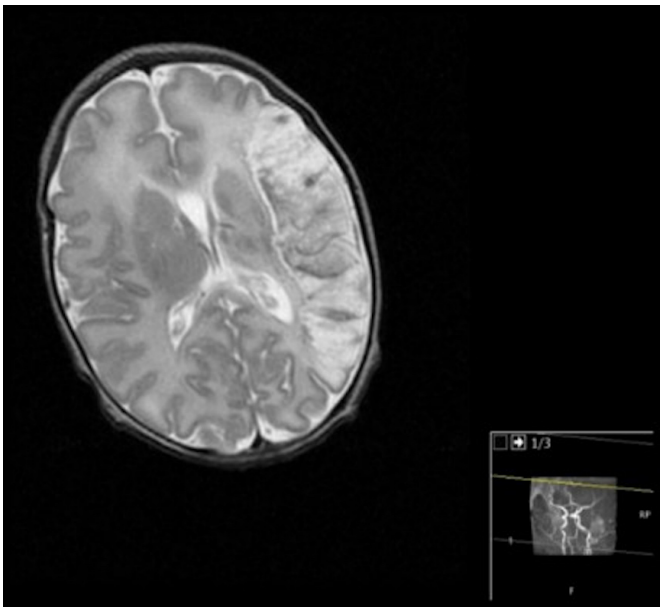

Fig 1. *Ischemic Stroke (MCA territory) at day twelve of life- MRI T2 with an extensive lesion on left frontotemporoparietal lobes and basal ganglia.*

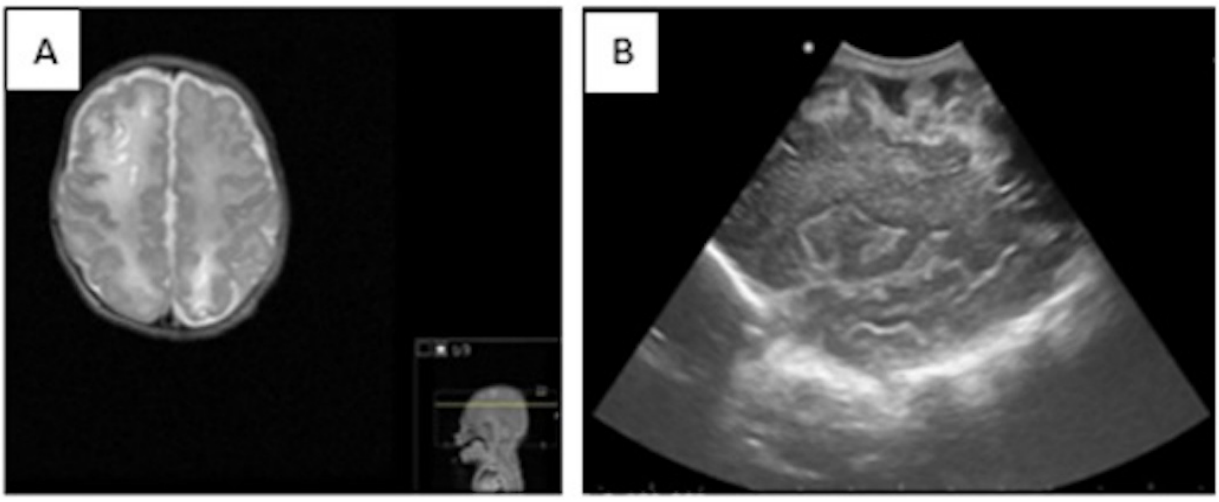

Fig. 2. *Ischemic Stroke (Watershed) at day 10 of life.* ***(A)*** *MRI T2 Ischemic lesions involving vascular border zones (right frontal and left parietal subcortical areas supplied by distal vessels of major arteries).* ***(B)*** *Cranial US: right parasagittal scan with hyperechogenicity of the right parietal convexity with cortex damage.*

Risk Factors

Most of the identified risk factors occurred in AIS group. The recognized risk factors identified in AIS group and HS group are listed in Table 2.

Table 2. *Ischemic and hemorrhagic risk factors´ data.* [4,9,12,13]

Risk Factors/ Stroke subtype	Ischemic (n=13) n (%)	Hemorrhagic (n=4) n (%)	Total (n=17) n (%)
Prenatal			
Intrapartum fever >38°C *	2 (15)	2 (50)	4 (24)
Oligohydramnios *	1 (8)	1 (25)	2 (12)
Pre-eclampsia *	1 (8)	-	1 (6)
Primiparity *	8 (62)	3 (75)	11 (65)
Perinatal			
Forceps or vacuum delivery *	3 (23)	1 (25)	4 (24)
Abnormal CTG*/Fetal distress†	3 (23)/-	-/-	3 (18)
Placental abruption †	1 (8)	-	1 (6)
Cord abnormalities *	1 (8)	-	1 (6)
Emergency CS *	3 (23)	-	3 (18)
Meconium-stained AF *	2 (25)	-	2 (12)
Neonatal			
Male *	9 (69)	4 (100)	13 (76)
Sepsis †	2 (15)	1 (25)	3 (18)
Resuscitation at birth *	2 (15)	-	2 (12)
5-min Apgar score <7 *	1 (8)	-	1 (6)
Hypoglycemia *	3 (23)	-	3 (18)
Hemostatic abnormalities †	1 (8)	-	1 (6)

All AIS cases had at least one specific AIS risk factor and just one case of all HS cases (25%) had a specific HS risk factor (neonatal sepsis).

Prothrombotic study was performed in three cases (18%): two AIS and one HS, one of which had an abnormal result - an AIS case with a heterozygous mutation G20210A on prothrombotic gene – a term neonate born by an emergent CS (fetal distress) whose father had the same mutation.

Placental histologic evaluation was performed in nine cases (eight for AIS and one for HS cases). Four placentas (50% of AIS cases) had abnormalities: chorioamnionitis/funisitis (two cases), thrombus (one case) and unspecific lesions (one case). Chorioamnionitis and funisitis occurred in a case of maternal fever; thrombus occurred in a fetal hydrops' case; unspecific lesions were found in a case of placental abruption.

Treatment And Outcome

During hospitalization 13 cases (76%) needed at least one anti-epileptic and four (31%) needed an association of two anti-epileptic (75% of AIS; 25% of HS). No cases received anticoagulation therapy. At the sixth month after event, the frequency of anti-epileptic therapy was higher in HS group; on the contrary, at the eighteenth month it was higher in AIS group (Table 3).

Table 3. *Follow-up and outcome in post-stroke infants.*

Follow-up and outcome / Stroke subtype	Ischemic (n=12*) n (%)	Hemorrhagic (n=4) n (%)	Total (n=16) n (%)
AE therapy	7 (58)	3 (75)	10 (62)
At 6th month	7 (58)	3 (75)	10 (62)
At 18th month	5 (42)	1 (25)	6 (38)
Neurologic sequelae	7 (58)	3 (75)	10 (62)
Epilepsy only	1 (8)	2 (50)	3 (19)
Hemiparesis only	4 (33)	-	4 (25)
Speech problems only	-	-	-
Epilepsy + Hemiparesis	1 (8)	-	1(6)
Speech problems + Hemiparesis	-	1 (25)	1 (6)
Epilepsy + Speech problems	1 (8)	-	1 (6)
ND evaluation	11† (92)	4 (100)	15† (94)
Normal	4 (36)	3 (75)	7 (44)
Abnormal	7 (64)	1 (25)	8 (50)

AE = Anti-epileptic drugs, ND = Neurodevelopmental

* One stroke case was lost to follow-up at four months of age. Anti-epileptic drugs and neurologic sequelae results included the lost case.

† One case had not available neurodevelopmental evaluation. Neurodevelopmental total results only include the children evaluated.

There was no recurrence of PS or death during this period.

The mean and median follow-up period were, respectively, 43 and 39 months (ranging from 18 to 80 months) for AIS and HS. One case was lost to follow-up at four months of age.

Neurological sequelae were present in 62% (58% AIS e 75% HS) of the 16 NB evaluated and are summarized in Table 3. Most common problems were hemiparesis (25%) and epilepsy (19%). One HS case developed refractory epilepsy and performed hemisferectomy at two years of age. Post stroke epilepsy incidence was similar at six and 18 months in both groups among patients who remained on anti-epileptic treatment. Speech problems were found in three cases: one AIS of the left MCA territory and two HS, at the ages of 63, 45 and 49 months respectively.

All AIS cases with more than two AIS risk factors had an abnormal neurologic outcome (Figure 3).

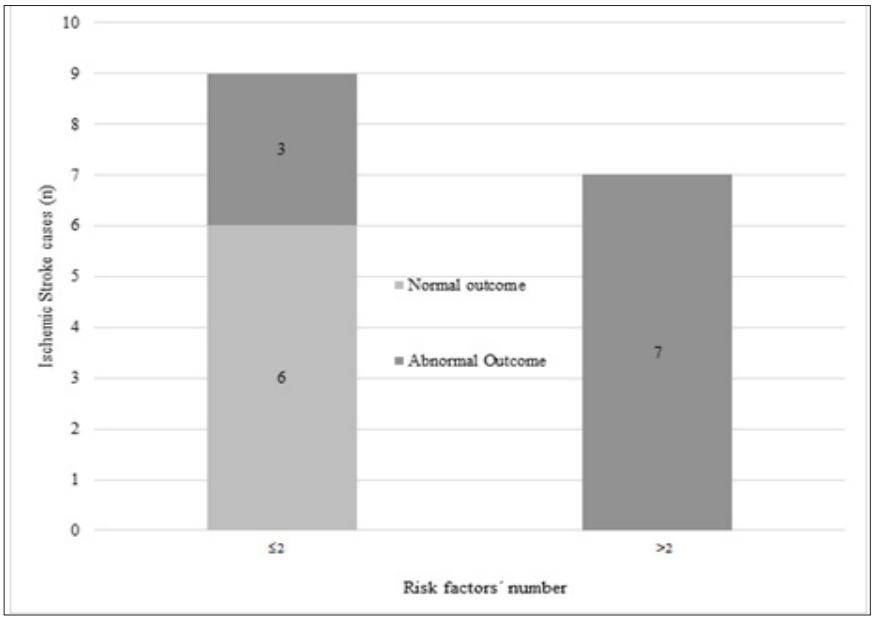

Fig 3. *Graphical representation of neurologic outcome based on AIS risk factors number in AIS group.*

The only HS case with a specific HS risk factor had an abnormal neurologic outcome (Figure 4).

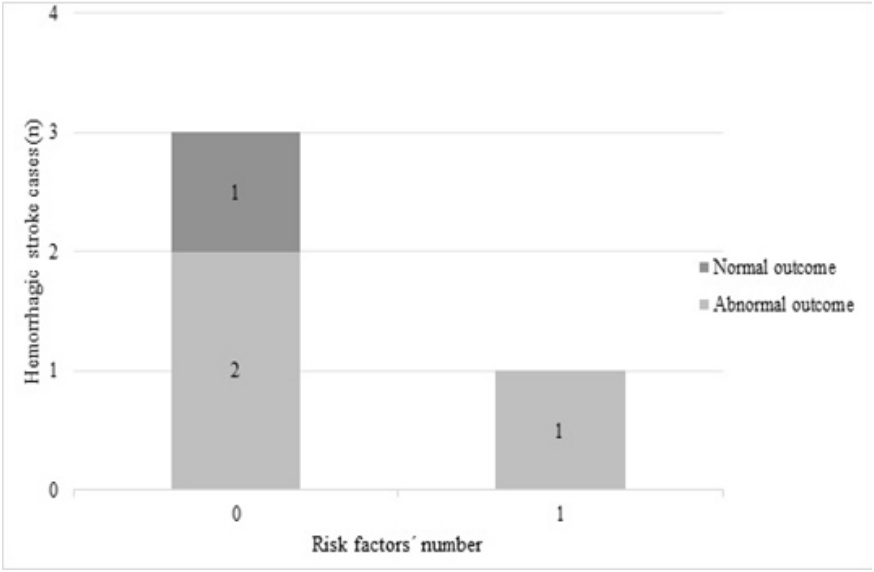

Fig 4. *Graphical representation of neurologic outcome based on HS risk factors number in HS group.*

From the 16 PS cases´ which had follow-up medical appointments, only in one case the neurodevelopmental evaluation was not available. Out of the 15 cases evaluated, 8 (53%) showed an abnormal neurodevelopmental evaluation (Table 3).

Discussion

Perinatal stroke incidence (one in 1872 live births) in this case series was higher than the incidence already described.[2] In agreement with previous studies, IS was the most common subtype of PS.[12]

As Lee et al. described in a 2017 published study, we confirm the predominance of male sex and full-term neonates in this PS case series.[6]

Seizures were the most common manifestation and presented in both AIS and HS groups, being consistent with previous studies which cite an incidence up to 92%.[6] Therefore, a differentiation of the two PS subtypes seems to be difficult based exclusively on clinical features. On the other hand, one should keep high level of suspicion if infants develop unexplained

seizures. The time between symptoms and confirmed diagnosis had an average of seven and six days for AIS and HS, respectively. The later diagnosis which occurred in two asymptomatic cases was insufficiently to consider this fact responsible for a poorer neurologic outcome, not confirming that previously described in literature.[3]

Besides having a significant impact in understanding PS pathogenesis, extensive prothrombotic screening and placental studies were only performed in specified cases, reflecting the already controversy published statements on this subject.[13]

Pathologic examination of the placenta was performed in a limited number of patients. It possibly indicates the different professionals´ attitude on the placentae selection, given the unspecificity of PS risk factors. On the other hand, as clinical manifestations of PS did not occur immediately after birth, many placentas can be unconsciously rejected. As prothrombotic and placental studies can have a significant impact in understanding PS pathogenesis, the need of a protocol in these two aspects seems to be of crucial importance.

The lack of studies on risk factors for HS as well as the limited case number collected in the present are important limitations. They might be partly responsible for having failed to detect specific risk factors for HS.

The presence of the same risk factors in HS and AIS cases´ could suggest common pathological pathways.

Mortality rate compared to that reported by Lee et al. (around 5%) may be justified by the lower number of stroke cases included in this single tertiary hospital-based study.[6] On the other hand, morbidity rate was significant – 62% of infants developed neurological sequelae. Nevertheless, the rate of motor dysfunction, speech problems and post-stroke epilepsy was lower to data previously reported.[6,7,15] The possible occurrence of serious long-term sequelae in PS, as reported by Amlie-Lefond and Ojemann, occurred in one case with intractable epilepsy requiring neurosurgery.[12]

Speech problems were only detected in patients after 45 months, and the number of affected children can be underestimated by the wide range of ages of follow-up assessments.

The association between risk factors´ number and a worse neurologic outcome in AIS was observed. As PS seems to be a multifactorial etiologic condition, the presence of multiple risk factors could reflect the presence of different etiologic factors, contributing to cumulative risk and increased neurologic impairment.

The main limitations of this study were the retrospective design and the limited number of patients (especially in the hemorrhagic group), based on a single tertiary hospital.

Conclusions

The present study reinforces the importance of a closer follow-up of post-stroke NB (regarding epilepsy and physical and neurocognitive acquisitions), suggesting a special attention in those cases with a combination of risk factors. An earlier supportive and rehabilitation intervention strategy in these high-risk NB could improve neurological outcome and provide a better life quality for children and their families. Important advances in the field of neonatal neurology were supported by the number of recognized risk factors for PS identified in this work, but in cases of HS we were still unable to demonstrate at least one risk factor. Much less is known about HS risk factors comparing to AIS risk factors, supporting the need of more research in this area. Also with few available data in literature, hemodynamic changes of neonatal period (transition from fetal to neonatal circulation), namely prothrombotic vascular changes of placenta (bi-directional shunt with movement of possible thrombus from the cord to left MCA of the NB), should deserve special attention.

Further prospective, human and animal studies are warranted to improve our knowledge about the mechanisms and timing of neurologic lesions establishment, verify possible common pathological pathways of specific risk factors, and confirm the potential contribution of risk factors´ combination to a poorer neurologic outcome.

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NEONATAL HYPOXIC-ISCHAEMIC ENCEPHALOPATHY: MOTOR IMPAIRMENT BEYOND CEREBRAL PALSY

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Neonatal hypoxic-ischaemic encephalopathy: Motor impairment beyond cerebral palsy

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ABSTRACT

Background: Research investigating neuromotor function in the absence of cerebral palsy (CP) for children who had neonatal HIE is limited.
Aims: To investigate school-age neurological and neuromotor function, and correlations with attention, neonatal Magnetic Resonance Imaging (MRI), and neuromotor assessments at toddler age.
Methods: Twenty-seven children with neonatal HIE without CP who underwent hypothermia treatment and a comparison group of 20 children were assessed at age 5–7 years for Minor Neurological Dysfunction (MND; simplified Touwen), motor skills (Movement Assessment Battery for Children-2; MABC-2), parental concern over motor function (MABC Checklist), general cognition (Wechsler Preschool and Primary Scale of Intelligence-IV, WPPSI), and attention (DuPaul ADHD Rating Scale). Neurological examination and motor development, using Bayley-3 scales, at age 24-months was extracted from the clinical database. Clinical neonatal MRI was assessed for hypoxic-ischaemic injury.
Results: In the HIE group, MND was more prevalent ($p = 0.026$) and M-ABC performance (total score $p = 0.006$; balance subtest $p = 0.008$) was worse; parents were more concerned about children's motor function ($p = 0.011$). HIE group inattention scores were higher ($p = 0.032$), which correlated with lower MABC-2 scores ($rs = -0.590$, $p = 0.004$). Neurological examination at 24-months correlated with MND ($rs = 0.437$, $p = 0.033$); Bayley-3 motor scores did not correlate with M-ABC-2 scores ($rs = 368$, $p = 0.133$). Neonatal MRI findings were not associated with school-age MND ($rs = 0.140$, $p = 0.523$) or MABC-2 ($rs = 0.300$, $p = 0.165$).
Conclusions: Children with neonatal HIE, without CP, treated with hypothermia may be more likely to develop MND and motor difficulties than typically developing peers. Inattention may contribute to motor performance. In the absence of CP, neonatal MRI and toddler age assessment of motor development have limited predictive value for school-age outcome. Since this was an exploratory study with a small sample size, findings should be confirmed by a definite larger study.

1. Background

Neonatal hypoxic-ischemic encephalopathy (HIE) is a condition which occurs in approximately 1.5 per 1000 live births in high income countries and more frequently in middle- and low-income countries [1,2]. Clinicians had little to offer until therapeutic hypothermia (TH) emerged. Several large randomised controlled

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trials have shown that TH reduces both mortality and severe neurodisability (Cerebral Palsy, CP), and these effects appear to continue to school age [3]. Commonly, children are discharged from neurodevelopmental follow up at 2 years of age if there is no global developmental or severe neuromotor impairment [4].

However, there is evidence that there is still increased risk for minor neurological, cognitive, or behavioural dysfunctions long term [5]. These typically go unnoticed in early life and manifest at late preschool or school age. Although they may be subtle, they are likely to impact real life functionality. Overall, long term outcome information about children with neonatal HIE who underwent TH and survived without developing CP is still relatively sparse. Most data come from the period prior to TH, and it is currently not clear whether findings from these studies hold for children treated with TH. In the TH era, a recent study by Jary et al., in school aged children showed that 38% of children who survived HIE following TH without CP had a Movement Assessment Battery for Children-2 (MABC-2) score of $\leq 15^{th}$ centile [6]. We have previously shown for a large clinical cohort who underwent TH, that at age 2 years, 12% of children without CP had minor neurological signs (gross or fine motor coordination difficulties, muscle tone imbalance) and this was associated with poorer motor, cognitive, and behavioural function compared to those with normal neurological examination [7].

Minor neurological dysfunction (MND), typically diagnosed at school age, is a description of the neurological profile of a child without CP or major impairments, which describes difficulties with posture, muscle tone regulation, balance, fine manipulative ability, mildly abnormal reflexes, movements, coordination, or cranial nerve function [8]. It can be classified as simple MND or complex MND, depending on the number of dysfunctional domains. Simple MND has limited clinical significance and may represent a normal suboptimal variant of neuromotor development. Complex MND is considered a form of brain dysfunction associated with neuroanatomical deficits and functional impairments such as learning disorders and behavioural problems [9]. Applying the concept of MND to children with neonatal HIE who have not developed CP, appears an attractive approach to stratify children's risk and target interventions.

Neonatal HIE is associated with brain injury to basal ganglia, thalamus, cortex, watershed zones, hippocampus, or brainstem, and the pattern of injury is dependent on the severity and duration of ischemia and hypoxia, and may correlate with specific neurological impairments [10]. Neonatal MRI is often used for prognostication of neurodevelopmental outcome following HIE, but the value in the context of an absence of CP is currently not well established [11], and findings are inconsistent. For example, in a study by Barnett et al., in children with neonatal HIE, not treated with TH, MND and lower motor scores (assessed with Touwen examination and MABC) were both associated with basal ganglia lesions (focal lesions affecting posterior lentiform nuclei and ventrolateral nuclei of the thalamus, without involvement of the posterior limb of the internal capsule [PLIC]) or focal signal abnormalities in white matter [12]. In contrast, in a study by Perez et al. [13], also in children who were not treated with TH, neonatal MRI pattern did not correlate with motor performance on the Zurich Neuromotor Assessment in children without major disability.

In this exploratory study, we want to investigate neurological examination and motor function, and possible correlations with general cognitive abilities and attention abilities, at school age in children with neonatal HIE who were treated with TH and survived without developing CP. We also aimed to assess whether neurological examination and motor assessments at toddler age correlated with school age outcomes. Finally, we aimed to explore possible correlations of neonatal clinical MRI and school age neurological examination and motor outcomes. We aimed to detect any differences and correlations which may be investigated and confirmed by future larger studies.

2. Materials and methods

Ethics approval for the study was given by the NHS Health Research Authority National Research Ethics Service Ethics Committee North West – Lancaster REC reference 15/NW/0292. Written informed consent was obtained from the parents, and assent from the children.

2.1. Participants

Participants were recruited from a clinical cohort of 95 children who had been admitted for consideration of TH for neonatal HIE at University Hospital Southampton, Southampton UK, a tertiary centre, between 01/08/2009 and 31/05/2013. The criteria for TH were gestational age $\geq$36 weeks and at least one of the following: Apgar score of 5 or less 10 min after birth; continued need for resuscitation 10 min after birth; or acidosis (defined as pH < 7 or base deficit >15 mmol/L, or both, in umbilical cord blood or any blood sample within 1 h of birth), signs of moderate to severe encephalopathy (classified using the modified Sarnat and Sarnat staging [14]). After exclusion of infants for whom, after admission, it was decided TH was not indicated, those who had a syndromic or chromosomal disorder, or encephalopathy as a consequence other than perinatal asphyxia, a sample of 70 newborns remained. Fifteen infants died in the neonatal period, leaving a sample of 55 infants who survived. No information after hospital discharge was available for 12 infants, leaving 43 children. Children who had a diagnosis of CP (n = 7), were not eligible for this study, 43 children who were eligible to take part. Five had moved out of the region, and of those who could be contacted, 31 families agreed to participate. Three children were excluded after recruitment since it emerged that they had a neurodevelopmental disorder not caused by perinatal asphyxia; one child was excluded since they had an incomplete assessment. The final sample was 27 children.

Supplementary Table 1 compares the subsample that formed the sample of this study and the whole cohort born and treated between 01/08/2009 and 31/05/2013. There were no significant differences in demographic or neonatal characteristics, i.e. this subsample can be regarded as representative of the whole cohort who underwent TH for neonatal HIE in that time period.

For the school age assessments, a comparison group was recruited using a friends and family approach and from local schools. The recruitment strategy was to achieve a comparison group of typically developing children of comparable age, sex, and post code area. Children who were on the Special Educational Needs Register were excluded.

2.2. Procedures and assessments

Children were invited for a follow-up visit at school age (5–7 years). The assessments included a neurological examination and assessment of motor skills, general cognitive abilities, and attention, as well as parents' views on motor/physical functioning in daily life (Fig. 1). For the children with HIE, information on routine clinical neurological and developmental assessments at age two years was retrieved from the clinical database, and clinical neonatal MRI was assessed for signs of hypoxic-ischaemic brain injury. The assessors were not blinded to whether or not the child had HIE.

2.2.1. Minor neurological dysfunction

A simplified version of the Touwen examination was used for

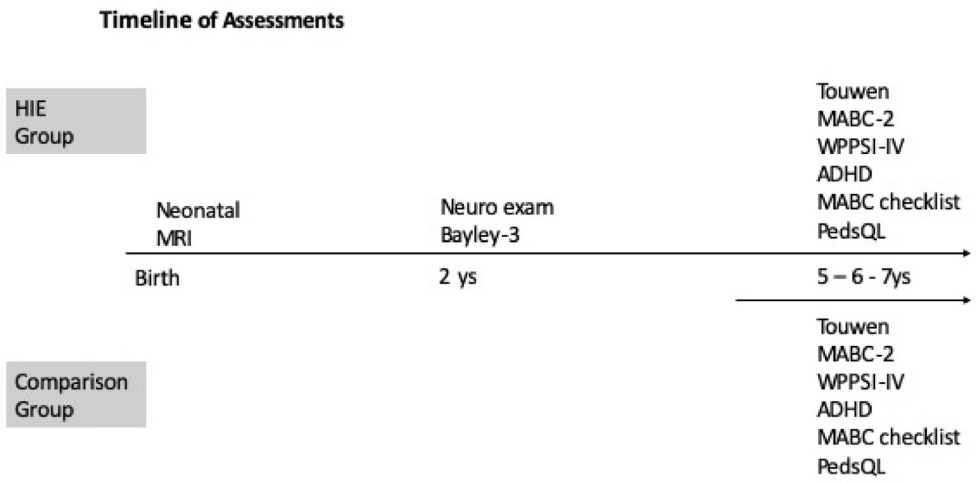

Fig. 1. Timeline of assessments in the HIE and comparison groups. Touwen examination (Touwen) was used for the assessment of Minor Neurologic Dysfunction (MND). Movement Assessment Battery for Children, Second Edition (MABC-2) was used to assess motor function. MABC Checklist (MABC Checklist) and the Measurement Model and Pediatric Quality of Life Inventory (PedsQL) — Parent Proxy Report Edition were applied to infer the real-life impact of motor dysfunction. Wechsler Preschool and Primary Scale of Intelligence 4thEdition (WPPSI-IV) was used to assess general cognitive abilities and children's attention was evaluated with the DuPaul ADHD Rating Scale Home and School version (ADHD). In HIE group, at 2-year-old, routine neurological examination (Neuro Exam) and assessment with the Bayley Scales of Infant and Toddler Development-3 (Bayley-3) were performed.

the assessment of MND [15]. This included the evaluation of four domains: reflexes, nerve function of the face and eyes, posture and muscle tone, coordination and balance. The findings were classified on the basis of the number of dysfunctional domains, as normal neurological function, simple MND (one or two domains abnormal), or complex MND (more than two domains abnormal).

2.2.2. Motor function

The Movement Assessment Battery for Children, Second Edition (MABC-2) was used to assess motor function [16]. MABC-2 includes three scales: manual dexterity, aiming and catching, and balance. For each of the three scales a standard score is calculated, and a total score is calculated by adding the scores from each subscale. For each scale and the total test score, centiles can be derived, and scores between the 5th-15th centile are indicative of borderline motor problems, scores below the 5th centile are indicative of definite motor problems.

2.2.3. Parents' view of children's everyday physical functioning

The MABC Checklist and the Measurement Model and Pediatric Quality of Life Inventory (PedsQL) — Parent Proxy Report Edition were used to infer the real-life impact of motor dysfunction according to parents' perception [16,17]. For this study, the Physical Functioning scale which comprises 8 items, was used. These items include questions on difficulties with walking, running, and activity levels. Scores are transformed on a scale from 0 to 100 and a higher score indicates a better health related quality of life.

2.2.4. General cognitive abilities

General cognitive abilities were assessed with the Wechsler Preschool and Primary Scale of Intelligence 4th Edition (WPPSI-IV) [18]. The mean index and IQ scores are 100, with a standard deviation of 15. The Visual Spatial Index (VSI), Verbal Comprehension Index (VCI), and Full-Scale IQ (FSIQ) score were used in this study.

2.2.5. Attention

Children's attention was assessed with the DuPaul ADHD Rating Scale Home, i.e. parents, and School version, i.e. teachers. The DuPaul ADHD Rating Scale is an 18-question self-report assessment with 9 items that ask questions about behaviour related to inattention [19]. As recommended by the manual, we used the raw scores for statistical analysis to increase uniformity and precision.

2.2.6. Neurodevelopmental assessment at age 24 months

Children with neonatal HIE underwent routine clinical neurological and developmental assessments at age two years (mean 27.3, SD 3.4 months). Information on neurological examination and assessment with the Bayley Scales of Infant and Toddler Development-3 (Bayley-3) were retrieved from the clinical database. Children had been assessed by a Paediatric Neurologist or Neonatologist with experience in neurological and developmental assessments, together with a Physiotherapist. Examiners were not blinded to neonatal course. Neurological examination included assessment of cranial nerve function, movements, posture, reflexes, and muscle tone. Neurological status was categorised as normal (completely normal neurological status), minor neurological signs (gross or fine motor coordination difficulties, muscle tone imbalance, without definite signs of cerebral palsy [CP]), or abnormal (signs of CP present as defined by the Surveillance of Cerebral Palsy in Europe Working Group, SCPE, 2000) [20]. The Bayley-3 is a standardised assessment which consists of a series of developmental play tasks [21]. Composite scores are derived for cognitive, language, and motor development, and scaled to a metric, with a mean of 100, standard deviation of 15, and range of 40—160. For this study we only used the motor scales.

2.2.7. Neonatal MRI

Information from clinical routine MRI studies was used, which was available for 23 out of the 27 children with HIE, performed at a median of 7 days (min 4 day, max 15 days) after birth. Infants were scanned on a 1.5 T scanner (Siemens Symphony, Siemens AG, Erlangen, Germany); T1-weighted, T2-weighted, and diffusion-weighted MRI images were used. MRI signs of neonatal hypoxic-ischaemic brain injury include signal abnormalities and/or diffusion abnormalities in typically affected brain structures (such as basal ganglia, thalami, perirolandic cortices and watershed areas) were assessed using a scoring system based on Barkovich, 1998 [22]. In addition, myelination of the posterior limb of the internal capsules (PLIC) was noted. Furthermore, if the thalami, caudate nuclei, putamina, globus pallidi, PLICs, and hippocampi showed

signal abnormalities (including diffusion abnormalities) on any of the MRI sequences, this was noted separately (binary score: affected vs. not affected). This is an in house developed scoring system and it is an attempt to detect more subtle grey matters injuries and/or in locations not specifically specified in the Barkovich scoring system (in which only thalamus and lentiform nucleus are specified as individual structures).

2.3. Statistical analysis

Data were assessed for normality by the Kolmogorov–Smirnov test. The Student's t-test and Mann–Whitney *U* test were used, as appropriate, to identify differences between the groups in continuous data. The Chi-square test or Fisher's exact test were used, as appropriate, to identify differences between the groups and investigate correlations between neurological classification groups and outcomes for categorical data. Spearman's correlation tests were used for correlations between neurological classification groups and continuous and discrete variable outcomes. The statistical significance level was set at a two-sided p value of less than 0.05. Uncorrected p values were used for this exploratory study, accepting the increased risk of type I errors in exchange for a higher sensitivity in detecting potential group differences and correlations.

3. Results

3.1. Demographics

Twenty-seven children with HIE without cerebral palsy (CP) were included in the study, along with 20 typically developing children ("comparison group"). The clinical characteristics for both groups are shown in Table 1. Mean age at assessment was significantly different ($p < 0.001$) between the groups, but no significant differences in distribution of sex between groups were seen ($p = 0.080$). Hearing and visual impairment were more common in children with HIE; this did not affect children's ability to participate in the tests.

3.2. MND prevalence and neuromotor function

Of the 27 children with HIE, 22.2% of children presented with MND (4 with simple MND, 2 with complex MND). All 20 children in the comparison group had normal neurological examination.

Children with HIE scored significantly lower on total MABC-2 scores than controls (effect size [r] = -0.398, $p = 0.006$) and significantly lower scores were observed for the manual dexterity ($r = -0.428$, $p = 0.003$) and balance ($r = -0.449$, $p = 0.002$), but not for the aiming and catching ($r = -0.087$, $p = 0.552$) subtests (Table 2); this is shown in Fig. 2. Children with HIE had a higher prevalence of clinically significant motor impairment: 6/27 (22%) children with HIE scored below the 15th centile in the total MABC-2 total score, compared with 1/20 (5%) in the control group.

3.3. General cognitive abilities

No significant group differences were found in FSIQ scores ($r = -0.181$, $p = 0.215$), VSI ($r = -0.223$, $p = 0.172$), or the VCI ($r = -0.129$, $p = 0.376$) scores of the WPPSI-IV (Table 2).

3.4. Parent and teacher rating for attention and motor function

Children with HIE scored significantly higher on the DuPaul ADHD Rating Scale (teacher version) Inattention subscale ($r = -0.353$, $p = 0.032$) than children in the comparison group, suggesting greater attentional impairment (Table 2). No such differences were found on the parent version of the ADHD Rating Scale. The parents of children with HIE were significantly more worried about their children's motor function than parents of children in the comparison group, as measured by the MABC Checklist ($r = -0.399$, $p = 0.011$). However, no difference was found in PedsQL Physical Functioning scores between the groups ($r = 0.040$, $p = 0.267$).

3.5. Correlations between MND, neuromotor function, and cognitive function

In the HIE group, there was a weak negative correlation between neurological status (MND) and total MABC-2 scores, suggesting that MND was associated with poorer motor functioning

Table 1
Clinical characteristics of children with a history of neonatal HIE and control children[a].

	HIE group, n = 27	Control group, n = 20
Sex, n male/female (%)	12/15 (44.44%)	4/16 (20.00%)
Age at assessment – mean (SD)	5 years 0 months (5.2 months)	5 years 7 months (5.8 months)
Gestational age	39.91 (1.43)	40.24 (1.12)
mean (SD) -weeks	37.00–41.86	38.00–42.00
min-max		
Birth weight	3432 (664)	3528 (595)
mean (SD) -g	2200–4980	2400–4590
min-max.		
Visual function - no. (%)	25 (92.60%)	13 (86.67%)
Normal:	1 (3.70%)	2 (13.33%)
Corrected with glasses:	1(3.70%)	0
Not fully corrected with glasses:		
Hearing – no. (%)	24 (87.11%)	14 (100%)
Normal:	1 (3.70%)	0
Corrected with hearing aids:	2 (7.40%)	0
Not fully corrected with hearing aids:		
Head circumference	49.73 (3.15)	51.29 (2.81)
mean (SD) – cm	37.00–53.00	46.00–58.00
min-max		

[a] Missing data: 2 HIE children missing head circumference. Control group information was missing for: visual function for 5 children and hearing function for 6 children; 6 missing head circumference; 9 exact gestational age (but all were born full term), and 8 exact birth weight (but all were born with appropriate for gestational age birth weight).

Table 2
Neuromotor, cognitive, and attention measures in the HIE and comparison groups.

	HIE group, n = 27	Control group, n = 20	p-values*	Effect size (r)
Motor function (MABC-2 total score)	8.96 (3.13)	11.45 (2.69)	0.006	0.398
Manual dexterity standard score (MABC-2 subtest)	8.44 (3.43)	11.25 (2.79)	0.003	0.428
Aiming and catching standard score (MABC-2 subtest)	9.07 (3.64)	9.70 (3.37)	0.552	0.087
Balance standard score (MABC-2 subtest)	9.85 (2.51)	12.55 (2.61)	0.002	0.449
Parental concern over motor function (MABC-2 Checklist; total score)	13.71 (13.80)	3.88 (2.58)	0.012	0.399
Full scale IQ (FSIQ score)	94.07 (12.52)	98.65 (11.59)	0.215	0.181
Visuo-spatial processing (VSI score)	89.89 (17.49)	97.11 (15.45)	0.169	0.223
Inattention (ADHD Rating Scale Teacher-Rated Inattention Score)	6.73 (7.11)	3.05 (4.90)	0.022	0.353
Inattention (ADHD Rating Scale Parent-Rated Inattention Score)	5.18 (4.99)	3.27 (2.68)	0.473	0.118
Physical functioning (PedsQL Physical Health Score)	83.62 (21.00)	92.29 (7.55)	0.253	0.181
2-year motor function (BSID-3 composite score)	108.78 (16.41)	-		
2-year fine motor function (BSID fine motor scaled score)	11.11 (3.05)	-		
2-year gross motor function (BSID gross motor scaled score)	11.78 (3.57)	-		

For each measure, mean scores and standard deviation are reported *p-values referring to Mann-Whitney *U* test results.

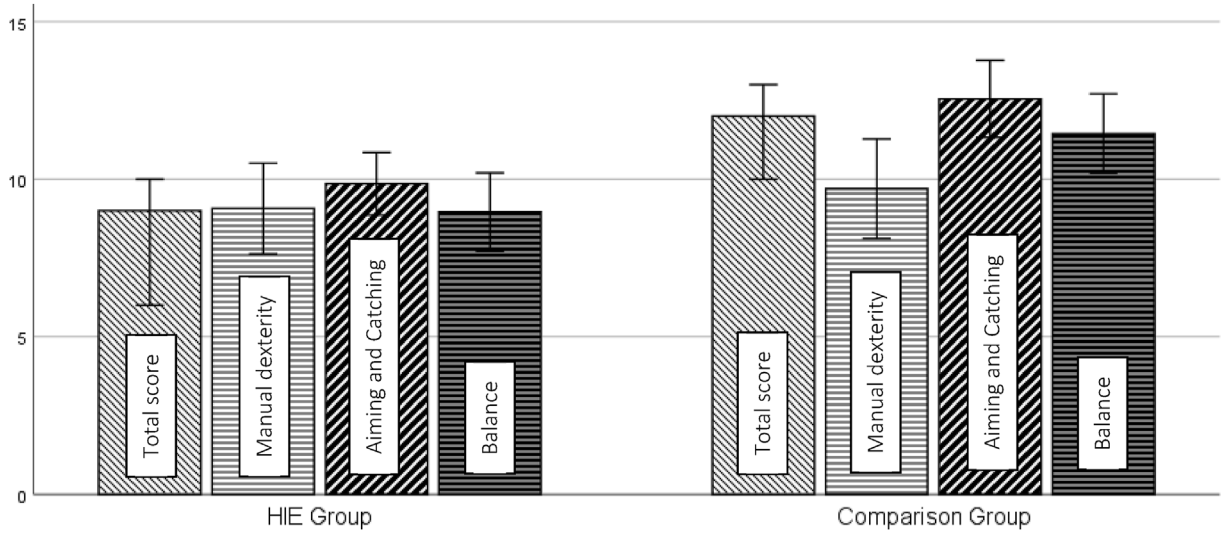

Fig. 2. Median values for MABC-2 total score and each MABC-2 subtest for children with HIE and children in the comparison group.

(rs = −0.364, p = 0.062). This was, however, limited to the balance subtest (rs = −0.396, p = 0.041); no correlation was found with the other subtests. There was also a weak-moderate negative correlation between MND and general cognitive abilities as measured by FSIQ, (rs = −0.482, p = 0.011) as well as a weak-moderate positive correlation between MABC-2 total and FSIQ scores (rs = 0.459, p = 0.016), indicating a correlation between cognitive and motor performance. This was dominated by manual dexterity (rs = 0.446, p = 0.020); aiming and catching (rs = 0.212, p = 0.277) and balance subtests (rs = 0.290, p = 0.142) were not associated with FSIQ. VSI scores did not correlate with overall MABC-2 scores (rs = 0.310, p = 0.196), nor with any of the subtests, suggesting that motor functioning in the HIE group was not significantly affected by their visual spatial processing skills. A weak correlation between lower VSI scores and MND was found, but this was not significant (rs = −0.415, p = 0.077). VCI scores correlated significantly with MABC-2 total scores (rs = 0.576, p = 0.002) and there was a strong, significant correlation between VCI and teacher-rated Inattention scores (rs = −0.748, p < 0.001) in the HIE group.

No correlations between neuromotor function, general cognitive abilities, or attention were seen in the comparison group.

3.6. Attention and neuromotor function

Teacher-rated ADHD Rating Scale Inattention scores showed a moderate negative correlation with total MABC-2 score (rs = −0.590, p = 0.004), as well as the balance subtest of the MABC-2 (rs = −0.567, p = 0.006); this is shown in Fig. 3. Furthermore, teacher ADHD Rating Scale Inattention scores were strongly correlated with FSIQ (rs = −0.712, p = 0.0002). Parent-rated Inattention scores also correlated with higher reported parental concerns about motor function, as measured by the MABC-2 Checklist, but this was not statistically significant (rs = 0.432, p = 0.057). No correlations were found between parent-rated Inattention scores and performance on the M-ABC.

3.7. Correlations between 24-month assessments and school age outcomes

Minor neurological signs at 24-months were weakly-moderately correlated with the presence of MND at school age (rs = 0.437, p = 0.033).

No correlation was found between Bayley-3 motor composite scores and MND or total scores on the MABC-2. However, lower scores in the gross motor and composite motor subtests of the Bayley-3 Scales correlated moderately-strongly with lower scores on the aiming and catching subtest of the MABC-2 (rs = 0.695, p = 0.001; rs = 0.663, p = 0.003, respectively). Lower scores in the gross motor and fine motor sections of the Bayley-3 correlated with increased parental concern over children's motor performance at

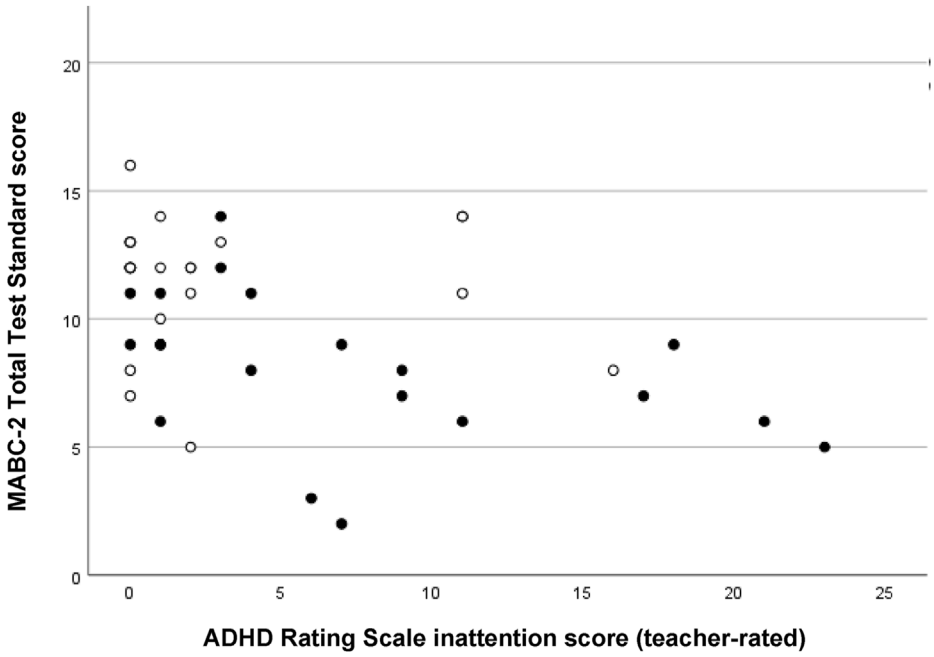

Fig. 3. – Correlation between MABC-2 total score and teacher rated Inattention scores for HIE and comparison groups Black dots represent children born with HIE White dots represent children from the comparison group.

school age, as measured by the MABC-2 Checklist ($p < 0.05$).

3.8. Neonatal MRI and school age neurological examination and motor outcomes

When applying the scoring system based on Barkovich et al. [22], neither Basal Ganglia score, nor Watershed scores, nor the combined Basal Ganglia-Watershed score was associated with any school-age outcomes. In 10 children, the Barkovich Basal Ganglia scored was 0, and, correspondingly, none of the grey matter structures nor the PLIC showed any signal abnormalities as assessed by the binary score. In the remaining 13 children, all had signal abnormalities in the thalami, 12 in the putamina, 10 in the globus pallidi or PLIC, 7 in the hippocampi, and 3 in the caudate nuclei. The grey matter structures followed the expected pattern of additional injury-involvement from thalami to the lentifrom nuclei, to the hippocampi [22] as well as caudate nuclei. However, only the 3 children with additional signal abnormalities in the caudate nuclei had significantly lower FSIQ scores ($r = -0.464$, $p = 0.016$) and VSI scores ($r = -0.559$, $p = 0.017$). Statistically non-significant correlations were seen in total MABC-2 scores ($r = -0.387$, $p = 0.066$), balance subtest scores ($r = -0.401$, $p = 0.052$) and VCI scores ($r = -0.393$, $p = 0.054$), with children with additional caudate abnormalities consistently scoring lower on all of these measures. In all children, the PLICs were normally myelinated and signal abnormalities in the PLIC, assessed by the binary score, were not associated with school age outcomes.

4. Discussion

In this small sample, MND, neuromotor difficulties, in particular manual dexterity and balance skills, and difficulties with inattention in the school setting, were more frequent in children with HIE than in the comparison group. MND was associated with poorer motor skills, and the prevalence of clinically significant motor impairment was higher in the HIE group, which was consistent with parental concerns about motor skills in daily life. Interestingly, although general cognitive abilities, measured by IQ scores, were similar in the two groups, in the HIE group, FSIQ scores were associated with MND and both, FSIQ and VCI scores with motor performance on the M-ABC, in particular in manual dexterity tasks. Of note, motor performance was not affected by visual spatial processing skills, as measured by VSI scores.

Prevalence of motor impairment at school age in the absence of CP in infants with HIE who underwent TH varies in the literature. In the TH arm of the NICHD hypothermia trial, Shankaran et al. reported that 5% of children who had TH and had not developed CP had gross and fine motor difficulties [23]. Jary et al., reported that 38% of their cohort of 29 children cooled for moderate/severe neonatal encephalopathy without CP had MABC-2 scores $\leq$15th centile [6]. In our sample 22% of cooled children without CP scored below the 15th centile on the M-ABC. Studies such as ours and that of Jary et al. can find variable rates of motor impairment, most likely due to the small sample sizes and the potential variation in the severity of neonatal encephalopathy.

Therapeutic hypothermia trials report variable IQ scores above or below the population mean at 6–7 years in cooled children with and without CP [24]. Lee-Kelland reported that school-age children cooled for HIE even in the absence of CP have significantly lower cognitive scores (IQ 14 points lower than their peers) [24]. In our sample, on a group level, children with HIE scored 5 IQ points lower on average than those in the comparison group, and lower FSIQ correlated with poorer neuromotor function, in particular with manual dexterity. Similarly, Jary et al. found significant correlations between MABC-2 manual dexterity scores and WISC-IV perceptual reasoning and working memory scores [6]. Their explanation for the difficulties of children with HIE in timed tests was related to impaired visuo-spatial and perceptual processing of instructions. In contrast, we did not find a relation between motor function and visual-spatial integration scores.

Motor and cognitive impairment in the HIE group was associated with attention problems in the school setting; this correlation was not present in the comparison group. Moreover, the correlation between higher inattention scores and lower verbal

comprehension scores could indicate that the correlations between poorer verbal comprehension and neuromotor deficits is compounded by attention problems. This is made even more likely by the fact that no between-group differences were found in VCI scores. Pre-clinical studies have demonstrated the emergence of attentional deficits after neonatal hypoxic and/or ischaemic events in animals. Tissue atrophy and dopaminergic disturbance in the pre-frontal cortex seems to be the reason for this correlation [25]. The pre-frontal cortex is responsible for the ability to plan and execute correct motor performance, an ability essential to the accomplishment of MABC tasks. In our sample, children with HIE with higher scores of inattention were more likely to have poorer motor performance, but also lower IQ scores, two functions in which prefrontal cortex is involved. In this way, our results are consistent with the preclinical data.

Parents of children with MND perceived their children to have motor problems in daily life. In addition, teachers' ratings on the ADHD scale suggest that children with HIE may display inattention in a school setting; inattention was associated with general cognitive and motor abilities, as well as with MND. This indicates that children with MND may be particularly affected at school. Interestingly, parents of children with HIE did not rate their children higher on the inattention scale than parents of children in the comparison group, suggesting that some of the difficulties reported may only be observed in specific daily activities settings. Detecting MND in children with a history of neonatal HIE but without CP may be a way to identify children with a high risk of future learning difficulties.

Minor neurological signs at 24 months were associated with MND at school age. However, gross motor developmental assessment at age 24 months was poorly predictive of motor skills at school age. The lack of a significant correlation between Bayley-3 performance and MND or performance on the MABC-2 have already been reported. Previous reports by Burakevych [26] and Jary [6] confirmed the poor predictive ability of the Bayley-3 for later motor skills. These findings reinforce the importance of serial assessment in children treated with cooling beyond the two years to at least school age, even in those with scores in the typical range at age two years. Additionally, the importance of identifying early minor neurological signs that may be predictive of minor neurological dysfunction in later life is highlighted by our findings.

Neonatal brain MRI provides prognostic information on death or severe disability beyond early infancy in HIE and therapeutic hypothermia does not appear to change its prognostic value [11]. A recent qualitative MRI injury scoring system weighted deep nuclear grey matter injury (thalamus and striatum) as significant predictor of neurodevelopmental outcome (defined as cerebral palsy, development delay and death) at 18–24 months in infants with neonatal HIE [27]. In our study, we found that children who had signal abnormalities within the caudate nuclei, in addition to signal abnormalities in the other deep grey matter structures, had significantly lower FSIQ and visual-spatial processing scores. This, however, must be interpreted with caution, as the number of children with caudate abnormalities was very small ($n = 3$). Poorer cognitive abilities in these children are probably the result of a more extensive brain injury rather than the isolated effects of abnormalities in the caudate nuclei.

We used a well established scoring system for MRI in the context of neonatal HIE [22], which considers basal ganglia, thalami, and cortex. However, it does not allow to explicitly focus on the caudate nuclei or the hippocampus, these structures are presumed to be included when assigning the scores that reflect more extensive injury to basal ganglia, watershed areas, or the cortex beyond the peri-rolandic region respectively. This may indicate a limitation of this commonly used scoring system when examining associations between brain injury and motor outcomes beyond cerebral palsy. However, injury to the caudate nucleus is of interest in the investigation of correlations between neonatal HIE and neurodevelopmental outcomes. The caudate nucleus has been demonstrated to be involved in cognition, visuo-spatial processing and motor function [28,29]. A recent study concluded that volume reductions in the caudate nuclei in children with HIE is associated with impaired motor coordination [30]. However, in that study, the sample was composed of different etiologies of neonatal encephalopathy, MRI was not done according to a standard protocol in the neonatal period, and children were assessed at later age. Future studies should aim to examine in more detail neural substrates for the neuromotor deficits observed in the neonatal HIE population, for example, explore anatomical and functional brain network integrity of the motor and cognitive systems.

Limitations of our work includes sample size and that we examined a clinical convenience sample. It is important to remember that our study was an exploratory one; a more definitive study in the future should ensure that multiple regression analysis is possible, which will require a larger sample. We chose to report uncorrected p values, despite having multiple comparisons and correlations. As an exploratory study, our aim was to detect potential differences between children born with HIE and their typically developing counterparts, as well as correlations between different test measurements. For this, type II errors must be minimised – something that would not be possible if Bonferroni correction were applied. When we repeated our analyses with Bonferroni correction applied, children born with HIE still scored significantly lower on the manual dexterity ($p = 0.03$) and balance subtests of the MABC-2 ($p = 0.02$). Greater levels of inattention in children born with HIE were still significantly correlated with lower FSIQ ($p = 0.008$) and VCI scores ($p = 0.009$) after correction for multiple analyses. A significant positive correlation between Bayley-3 gross motor scores at 24 months and performance on the aiming and catching subtest of the MABC-2 was also detected ($p = 0.04$). Thus, some of our results still remain statistically significant after the application of Bonferroni correction; however, as the purpose of this study requires the prioritisation of minimising type II errors over limiting type I errors, we focused on reporting uncorrected results. We recommend for these to be confirmed in larger scale studies where Bonferroni correction can be applied more readily.

This study also highlights the limitations of visual assessment of standard anatomical MRI sequences used in clinical routine as prognostic tools following HIE in the absence of CP. This study was performed at 1.5T MRI which is in line with concurrent studies at that time. Imaging at higher field strengths, i.e. at 3T in the first week, which is now common, might increase sensitivity to detect hypoxic-ischaemic lesions.

5. Conclusions

In the absence of CP, about 1/5 of children with HIE treated with TH had significant motor impairment at school age. This was associated with the presence of MND and parent reports indicate that this has implications for daily functioning. Motor function impairment was associated with general cognitive difficulties and attention problems at school. It is important to keep in mind that early neurodevelopmental assessments may be poor predictors of later motor function. Injury to the caudate nuclei may play a role in neuromotor and general cognitive deficits following neonatal HIE. Our findings emphasize the importance of long-term follow-up, which includes standardized assessment of neurology and motor function.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpn.2021.10.005.

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Corrigendum to "Neonatal hypoxic-ischaemic encephalopathy: Motor impairment beyond cerebral palsy" [Eur J Paediatr Neurol. 35 (2021) 74–81]

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The authors regret that the printed version of the above article contained an error, relating to Fig. 2. The correct and final version follows below. The authors would like to apologise for any inconvenience caused.

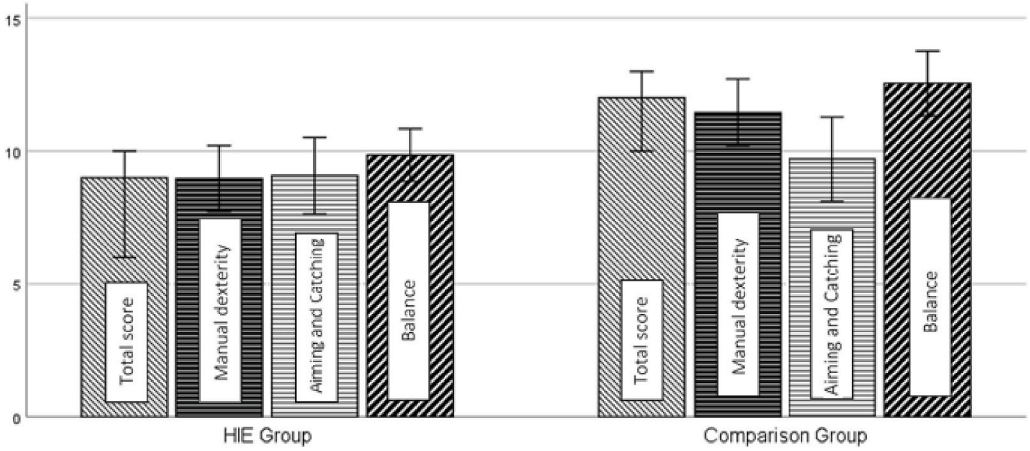

Fig. 2. Median values for MABC-2 total score and each MABC-2 subtest for children with HIE and children in the comparison group.
Error bars represent 95% confidence intervals.
The bars for each group represent, from right to left: 1 - MABC-2 Total Test Standard Score; 2 – MABC-2 Manual Dexterity Subtest Standard Score; 3 - MABC-2 Aiming and Catching Subtest Standard Score; 4 - MABC-2 Balance Subtest Standard Score.

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BEHAVIORAL AND BRAIN MORPHOLOGICAL ANALYSIS OF NON-INFLAMMATORY AND INFLAMMATORY RAT MODELS OF PRETERM BRAIN INJURY

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Behavioral and brain morphological analysis of non-inflammatory and inflammatory rat models of preterm brain injury

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ABSTRACT

Investigations using preclinical models of preterm birth have much contributed, together with human neuropathological studies, for advances in our understanding of preterm brain injury. Here, we evaluated whether the neurodevelopmental and behavioral consequences of preterm birth induced by a non-inflammatory model of preterm birth using mifepristone would differ from those after inflammatory prenatal transient hypoxia–ischemia (TSHI) model. Pregnant Wistar rats were either injected with mifepristone, and pups were delivered on embryonic day 21 (ED21 group), or laparotomized on the 18th day of gestation for 60 min of uterine arteries occlusion. Rat pups were tested postnatally for characterization of developmental milestones and, after weaning, they were behaviorally tested for anxiety and for spatial learning and memory. One month later, brains were processed for quantification of doublecortin (DCX)- and neuropeptide Y (NPY)-immunoreactive cells, and cholinergic varicosities in the hippocampus. ED21 rats did not differ from controls with respect to neonatal developmental milestones, anxiety, learning and memory functions, and neurochemical parameters. Conversely, in TSHI rats the development of neonatal reflexes was delayed, the levels of anxiety were reduced, and spatial learning and memory was impaired; in the hippocampus, the total number of DCX and NPY cells was increased, and the density of cholinergic varicosities was reduced. With these results we suggest that a preterm birth, in a non-inflammatory prenatal environment, does not significantly change neonatal development and adult neurologic outcome. On other hand, prenatal hypoxia and ischemia (inflammation) modifies developmental trajectory, learning and memory, neurogenesis, and NPY GABAergic and cholinergic brain systems.

1. Introduction

Preterm birth is defined as the birth before 37 weeks of gestation, and is regarded by the World Health Organization as one of the major health problems (Blencowe et al., 2013). Its incidence in most developed countries is about 5–7% of live births (Goldenberg, Culhane, Iams, & Romero, 2008), and the associated complications are responsible for approximately 35% of the world's neonatal deaths and for important morbidity in survivors (Blencowe et al., 2013). Approximately 25% of cerebral palsy cases result from preterm birth. Cerebral palsy or milder developmental coordination disorder, impairments of learning, memory, executive function, vision and hearing, epilepsy and psychiatric disorders can all be consequence of preterm birth (Soleimani, Zaheri, & Abdi, 2014). It has a complex aetiology, involving maternal, foetal and placentary causes, and even though various factors have been associated with increased risk of premature birth, there is no common pathophysiological explanation that contemplates all mechanisms involved (Muglia & Katz, 2010; Vogel et al., 2018).

The neurologic and psychiatric deficits found in preterm humans are globally explained by encephalopathy of prematurity, a concept introduced by Volpe that is characterized by an array of grey and white matter lesions that result from combined acquired insults, altered developmental trajectories and disorganized reparative phenomena (Volpe, 2009). Despite rapid advances in neuroscience research, the

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available knowledge about the pathophysiology of encephalopathy of prematurity is still insufficient. An important obstacle to elucidate the pathophysiology of this condition are the limitations of the preclinical models that only partially recapitulate the molecular, cellular, histological and functional abnormalities observed in humans (Jantzie & Robinson, 2015a). The most commonly used animal models induce in utero inflammation consequent to hypoxic-ischemic injury and/or infectious insults, but they do not mimic spontaneous preterm birth (Hagberg et al., 2015; Jantzie, Winer, Maxwell, Chan, & Robinson, 2015). Moreover, in many of these models, the injury occurs only in postnatal period of the rodent and, therefore, do not consider the influence of in utero environment as a contributor for foetal development. In the present work, we attempted to address this subject by comparing the effects on neonatal behavior and neurodevelopment later in life of a non-inflammatory, mifepristone-induced, preterm birth model that simulates spontaneous premature birth and an in utero inflammatory model of brain injury using transient hypoxia–ischemia. It is known that, in rats, term parturition occurs after involution of the corpus luteum and consequent decrease in serum progesterone levels (Elovitz & Mrinalini, 2004). To simulate spontaneous premature birth, we have used mifepristone, a steroidal antiprogestogen that, in the presence of progesterone, acts as a competitive progesterone receptor antagonist, thereby inducing a decrease in serum progesterone and birth occurs within 20–24 h after its administration (Dudley, Branch, Edwin, & Mitchell, 1996). Previous reports using mifepristone have focused in preterm parturition and few examined the effects on the newborn rat.

Our aim was to better understand the mechanisms and consequences of encephalopathy of prematurity. Specifically, to appreciate whether inflammation, via hypoxia–ischemia, is an essential trigger for the development of encephalopathy of prematurity or whether premature birth itself, in a non-inflammatory environment, can also contribute to the injury. To achieve our goal, we characterized the neurologic outcomes of preterm rats delivered after the administration of mifepristone (non-inflammatory model) and rats subjected to in utero exposure to a hypoxia–ischemia insult (inflammatory model). In this study rats were followed from the neonatal period to postnatal day 60 and assessed by neurologic and behavioral tests to examine locomotor activity, anxiety and learning and memory. Moreover, we analysed whether there is a relation between the potential behavioral alterations induced by these two preterm models and dentate gyrus neurogenesis using doublecortin (DCX), a marker of neuroproliferation. Knowing that neuropeptide Y (NPY)-positive interneurons are an important and representative population of gamma-aminobutyric acid (GABA)-ergic neurons of the hippocampus we decided to analyse if these treatments will interfere with the number of the NPY-positive interneurons. Furthermore, taking into account that the NPY-ergic system may be interdependent of the cholinergic innervation in the cerebral cortex (Milner, Wiley, Kurucz, Prince, & Pierce, 1997), we also decided to analyze the effects of prematurity and prenatal hypoxia–ischemia on the cholinergic system of the hippocampus.

2. Material and methods

2.1. Animals and preterm models

Wistar rats derived from the Institute for Molecular and Cell Biology (Porto, Portugal) were used. They were housed at a constant temperature (ambient temperature of 23 ± 1 °C, 45 ± 5% relative humidity) on a constant light/dark cycle (12:12), with lights on at 07:00 h and off at 19:00 h. Food and water were available ad libitum throughout the experiments. Starting at 3 months of age, female oestrous cycle was monitored daily by vaginal swabs. Females at proestrus were paired with an experienced male overnight. The following morning, the male was removed, and the mating outcome was checked by observation of a vaginal plug and by microscopic observation of sperm in the vaginal smear. If sperm was present, embryonic day (ED) 1 was noted. On ED18, females were equally divided into the following two experimental groups: (1) Non-inflammatory model – mifepristone-induced birth and (2) inflammatory model – transient systemic hypoxia–ischemia (TSHI). In the first group, the non-inflammatory group, later designated ED21, preterm parturition was induced by a single dose of 2 mg/kg body weight of mifepristone - RU 486 ((17ß-hydroxy-llß-(4-dimethylaminophenyl-l)-17a(prop-l-ynyl) oestra-4,9 dien-3one). The solution (0.5 ml) was injected subcutaneously in the left or right flank of the hind leg of each animal at 09:00 h on ED18, ED19 or ED20. Only pups from females injected on ED20 survived, and were born, as expected, 24 h later. Controls for this experimental group were injected, in the same site with 0.5 ml physiological saline; pups were naturally born on ED23.

In the second experimental group (TSHI group), females were treated following an already established model for inducing preterm transient systemic hypoxia–ischemia (Jantzie et al., 2015). Accordingly, on the ED18, females were anesthetized with sevoflurane (SevoFlo, Abbott Laboratories Ltd, Maidenhead, UK) and submitted to laparotomy, exposing the uterus and uterine horns. A 30G aneurism clamp was placed on the uterine arteries ensuring cessation of blood flow. After 60 min, the clamps were carefully removed and the peritoneal cavity thoroughly irrigated. Using forceps, the uterus and uterine horns were returned to the peritoneal cavity. To obtain an even distribution of pressure and abdominal distension for the pregnancy, the abdominal wall was closed using a two-layer continuous suture. Pups in this group were naturally born of ED23. In the control group, females were treated the same as in the TSHI group, except for transient uterine arteries ligation.

Dams (n = 3 per group) were housed individually and allowed to raise their own litters until weaning (4 weeks). Both male and female offspring were included in the study. After weaning, the offspring were housed in cages with three rats of same-sex, same-"time of birth" per cage. All pups (n = 30 per group, ~50% males) were monitored every 1 h for the first 24 h and every 2 h in the next 48 h. For the rest of the suckling time, animals were checked at regular times.

All experiments were carried out in accordance with the guidelines of the European Communities Council Directives of 22 September 2010 (2010/63/EU) and Portuguese Act n°113/13, and approved by ORBEA, the internal committee of the Faculty of Medicine, University of Porto, Portugal.

2.2. Developmental milestones and reflex development in pups

All developmental examinations and neonatal behaviors were performed during the light phase (between 09:00 h and 16:00 h) by two examiners. Monitoring of developmental milestones started on postnatal day (P) 6, in order to avoid disturbing the nest and affecting maternal behavior and continued until pups fulfilled all milestones. Neonatal reflexes and milestones were evaluated by applying a standard battery of tests that are summarized below with indication of postnatal days in which tests were performed (Baharnoori, Bhardwaj, & Srivastava, 2012; Lubics et al., 2005; Moser, 1999). Tests were classified as present or not and, when present, as the time spent to complete them.

2.2.1. Forelimb grasp reflex (P6–P10)

A steel rod of 1 mm was rubbed against the palm of the forepaw of the pups. The reflex was considered fully developed when the pups grasped the wire. The day the pups attained this milestone was recorded.

2.2.2. Cliff avoidance reaction (P6–P10)

The test was assessed by using a wooden platform (30 × 20 × 1 cm), located 70 cm above the floor. The floor below the platform was carpeted to prevent injury if the animal fell. The test was initiated by gently placing the animal on the platform with front paws and snout over the edge. If the animal fell from the platform, it was judged to have impaired cliff avoidance reaction. If the animal did not fell from the platform, the time required to retract from the edge and turn 180° away from the cliff

face was recorded.

2.2.3. Negative geotactic reaction (P6–P11)

The pups were placed head down, with hind limbs in the middle of an inclined surface (45°) of 30 cm length covered with wired mesh. Each pup was observed for 30 s to turn and move toward the upper end of the surface. The time spent and the day the pup was able to do it was recorded. If the pup did not turn around and climb up the board within the observed 30 s, the test was considered negative.

2.2.4. Proximal forelimb strength (P10)

The pups were encouraged to suspend themselves by their forepaws from a horizontal steel rod placed 40 cm above the floor. The floor below was carpeted to prevent injury when the animal fell. The time the pups stayed suspended was recorded in s.

2.3. Behavioral studies

All behavioral experiments were conducted after weaning (4-week-old rats) during the standard light phase, starting at 12:00 h, and were done by two experimenters blinded to the treatments. Before testing, animals were handled for 5 consecutive days. Experiments were performed after at least 30 min habituation of the rats to the testing room. Tests were performed, with 1-day inter-test intervals, in the following order: Morris water maze test, working memory test, open-field, elevated plus-maze and novel object recognition test.

2.3.1. Morris water maze - Spatial reference memory task

In order to assess spatial learning and memory, rats were tested in a black circular pool (180 cm diameter; 50 cm deep) filled with water at room temperature (21 ± 1 °C) that was located in a corner of a room containing extra-maze cues. The pool was virtually divided into 4 equal-size quadrants. A black escape platform (10 cm in diameter) was placed in the centre of one of the quadrants, 2 cm below the water surface. Swim paths were recorded by a computerized video-tracking system (EthoVision XT 8.5, Noldus, The Netherlands). In the place learning task, rats were trained to find the submerged escape platform and to climb on it. For acquisition, rats were given 2 trials per day for 14 consecutive days, as follows. The test rat was placed in the water facing the pool wall at one of four starting points, which were used in a pseudo-random order so that each position would be used just once in each block of 4 trials. When rats did not find the escape platform within 60 s, the experimenter guided them to the platform where they were allowed to remain for 15 s. After the first daily trial, rats were placed in a clean cage for 30 s before the beginning of the next trial. The platform location was not changed during the acquisition period. The swim path length in each trial was measured. One day after the end of the acquisition period, rats were submitted to a single 60-s probe trial, in which the platform was removed from the pool. The number of times the rats swam through the zone where the platform had been located (platform crossings), and the time spent by the rats swimming on the target quadrant were recorded. One day later, all rats were tested on the visible platform task to evaluate their sensorimotor abilities. In this task, rats were given 1 block of 4 trials separated by 30-s inter-trial intervals. The platform, painted in white, was exposed 3 cm above the water surface and its position was different in each trial. The distances swum to locate the platform were recorded and averaged across 4 trials.

2.3.2. Spatial working memory

Three days following completion of the reference memory task, rats were trained on a delayed-match-to-place version of the Morris water maze to assess the spatial working memory. During six consecutive days, rats received two trials per day. On the first trial (information trial), the submerged platform was located in a novel position, different in quadrant and distance from the edge of the maze from the placement on the previous days. Each rat was placed in the water facing the pool wall in the distal position from the platform. If the rats did not find the escape platform within 60 s, the experimenter guided them to the platform where they were allowed to remain for 15 s. On the second trial (retention trial), the submerged platform was located in the same position as the information trial. The start position was always distal from the platform. The trials were otherwise performed in the same manner as in the spatial reference memory task. A 1 min inter-trial interval was allowed between information and retention trials.

2.3.3. Open-field test

To assess general exploratory locomotion and anxiety-like behaviors, we used an open-field apparatus that consisted of a white acrylic arena (100 × 100 × 40 cm). The test rat was placed in a corner of the apparatus and tested during 5-min sessions. Distances travelled in the outer zone of the open field, defined as 20 cm from any wall, and in its inner zone, defined as the 60 × 60 cm square in the centre of the arena, were measured using a computerized video-tracking system (EthoVision XT 8.5, Noldus, The Netherlands). At the end of each session, the number of fecal boli deposited was counted and recorded, and the urine deposited was collected using a filter paper. The difference between the weight (in g) of the paper before and after collecting the urine was considered as a measure of the amount of urine deposited during the session. The floor of the apparatus was thoroughly cleaned and dried between each session.

2.3.4. Elevated plus-maze

To further evaluate general exploratory and anxiety-like behaviors, an elevated plus-maze apparatus consisting of a black acrylic cross with 2 opposite open and 2 opposite closed arms (50 × 12 cm) joined by a common central square (12 × 12 cm) was used. The closed arms were enclosed by 50 cm high walls. The test rat was placed on the central square, facing one of the closed arms and allowed to explore the apparatus for 5 min. The behavior of the rat was recorded and analysed using a computerized video-tracking system (EthoVision XT 8.5, Noldus, The Netherlands). The percentages of time spent, and the distances travelled by rats in the open arms, in the closed arms and in the central square were measured. At the end of each session, the number of fecal boli and the amount of urine were recorded. The apparatus was then thoroughly cleaned and dried.

2.3.5. Novel object recognition test

All testing was conducted in a 100 × 100 × 40 cm white acrylic arena and a computerized video-tracking system (EthoVision XT 8.5), positioned directly above it, was used. Objects to be discriminated were a black (7 × 7 × 7 cm) and a brown box with two white stripes (4 × 4 × 4 cm) made of glass. Rats were habituated twice for 10 min to the arena and boxes ensuring familiarity with the testing environment. On the testing day, each rat was placed in the corner of the arena and allowed to explore two undistinguishable black boxes for 5 min (trial phase). The rat was then returned to its home cage for a 2 h inter-trial interval. The arena and objects were cleaned with 70% ethanol. In the test phase, the rat was returned to the arena and allowed to explore a familiar (black box) and a novel object (a brown box with two white stripes) for 5 min. Familiar and novel objects were alternated, between animals, in the left and right positions to prevent location bias. Object exploration was defined as the rats sniffing or touching the objects but not by leaning against, standing on, turning around on or sitting on the objects.

2.4. Tissue collection and immunocytochemistry

At 60 days of age, 6 rats were randomly selected from each experimental group. Because we found no differences in the developmental milestones and behavioral tests between controls of the ED21 and TSHI groups, controls from both groups were pooled together. Rats were deeply anesthetized with sevoflurane (SevoFlo, Abbott Laboratories Ltd, Maidenhead, UK) and transcardially perfused with 150 ml of 0.1 M

phosphate buffer followed by a fixative solution containing 4% paraformaldehyde in phosphate buffer at pH 7.6. The brains were removed from the skulls, coded for blind processing and analysis, and separated by a midsagittal cut into right and left hemispheres. The frontal and occipital poles were removed and the remaining blocks of tissue containing the hippocampal formation were separated and processed for immunohistochemistry. Because prior studies had shown that the hippocampal formation of rodents displays right/left asymmetries (Slomianka & West, 1987), the blocks were alternately sampled from the right and left hemispheres.

The blocks containing the hippocampal formation were stored for 1 h in the fixative solution used in the perfusion and maintained overnight in a 10% sucrose solution at 4 °C. Blocks were then mounted on a vibratome, serially sectioned in the coronal plane at 40 μm and collected in phosphate-buffered saline (PBS). From each brain, three sets of vibratome sections containing the hippocampal formation were selected, using a systematic random sampling procedure. The first section of each section was randomly selected from the first group of 12 collected sections, and the remaining sections were sampled at regular intervals of 480 μm (i.e., 1 out of 12 sections) along the septotemporal extent of the hippocampal formation. The first, the second and the third series were used for doublecortin (DCX), neuropeptide Y (NPY) and vesicular acetylcholine transporter (VAChT) immunohistochemistry, respectively. Sections were washed twice with PBS, treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase and incubated overnight at 4 °C with the primary antibody against either DCX (Santa Cruz Biotechnology, 1:500 dilution in PBS), NPY (Bachem; 1:10,000 dilution in PBS) or VAChT (Chemicon; 1:15,000 dilution in PBS). After that, the sections were washed twice and incubated with the corresponding biotinylated secondary antibody (Vector Laboratories; 1:400 dilution in PBS). Sections were then treated with avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories; 1:800 dilution in PBS). In the two last steps, the incubation was carried out for at least 1 h at room temperature. Following treatment with the peroxidase complex, sections were incubated for 10 min in 0.05% diaminobenzidine (Sigma) to which 0.01% H_2O_2 was added. Sections were rinsed in PBS for at least 15 min between each step. To increase the tissue penetration, 0.5% Triton X-100 was added to PBS that was used in all immunoreactions and washes. Specificity of the immune reactions was controlled by omitting the incubation step with primary antisera; in these sections, no immunostaining was observed (data not shown) All immunochemical reactions and washings described above were carried out in 12-well tissue culture plates, 4 sections in each well, to assure that staining of the sections from all groups analysed was performed in parallel and under identical conditions. Following the staining procedures, sections were mounted on gelatine-coated slides and air-dried. They were then dehydrated in a series of ethanol solutions (50%, 70%, 90% and 100%) and coverslipped using histomount (National Diagnostics).

2.5. Morphometric analysis

2.5.1. Estimation of the total number of DCX- and NPY-immunoreactive cells

The total number of cells were estimated using the optical fractionator method (West, Slomianka, & Gundersen, 1991). The estimates for DCX cells were obtained in the subgranular layer and in the hilus (for ectopic cells) of the dentate gyrus, and the estimates for NPY cells were taken in the hilus of the dentate gyrus and pyramidal strata of the hippocampal subfields CA1 and CA3. Cells were considered to be immunoreactive for DCX or NPY when they displayed darkly stained perikarya. The hilus and the layers were consistently delineated, at all levels along the septotemporal axis of the hippocampal formation, on the basis of cytoarchitectonic criteria (Witter & Amaral, 2004) and by using a Rat brain atlas (Paxinos & Watson, 1998); the subgranular layer was further defined as an approximately 30 μm thick ribbon of tissue between the granular layer and the hilus (McClain, Hayes, Morris, & Nixon, 2011). Cell counting was carried out using the Olympus C.A.S.T.-Grid System (Denmark) and a mean of 12 systematically sampled sections per animal. Beginning at a random starting position, visual fields were systematically sampled along the x- and y-axes, using a raster pattern procedure. Neuronal nuclei were selected as the counting unit. They were counted in every frame using the optical disector at a final magnification of ×2000, at the level of the monitor. The coefficient of error (CE) of the individual estimates ranged between 0.08 and 0.10 and was calculated as previously described (Andersen & Gundersen, 1999).

2.5.2. Estimation of hippocampal layers volumes

The volumes of the hippocampal layers were estimated using the principle of Cavalieri (Gundersen et al., 1988; Regeur & Pakkenberg, 1989). Estimations were carried out using the Olympus C.A.S.T.-Grid System (Denmark) and a mean of 12 systematically sampled sections per animal. In each section, the cross-sectional area of each cortical layer was estimated by point counting (Gundersen & Jensen, 1987), at a final magnification of 80×, using an adequate grid of test points. The volume of the layers was calculated from the total number of points that fell on each layer and the distance between the sections (Gundersen et al., 1988; Regeur & Pakkenberg, 1989). The CE of the individual estimates was calculated as shown by Cruz-Orive (Cruz-Orive, 1999) and the mean value was 0.04.

2.5.3. Estimation of the areal density of VAChT-positive varicosities in the dentate hilus

The cholinergic varicosities stained with VAChT were counted using a computer-assisted image analyser (Leica QWin) fitted with a Leica DMR microscope and Leica DC 300F video camera. For each animal, an average of 12 VAChT-stained sections were used and analysed as previous described (Cardoso, Paula-Barbosa, & Lukoyanov, 2006). Measurements were performed at a final magnification of 1000x. The varicosities were defined as darkly stained axonal dilations with size greater than 0.25 μm^2 (Wong, Debeir, Duff, & Cuello, 1999). A sample frame (3.86×10^3 μm^2) was laid over each field of view, and the number of varicosities falling within it was counted. Within each section, four different placements of the frame, each time at a randomly selected position, were used to obtain a mean count for the dentate hilus. The results were expressed as area densities (number/mm^2).

2.6. Statistical analyses

Behavioral results are expressed as mean ± SEM and morphological data as mean ± SD. Statistical analyses and graphics were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Repeated measures analysis of variance (ANOVA) was used to analyse data from mean time in negative geotaxis, cliff avoidance, forelimb grasp, distances travelled and escape latency in the Morris water maze, and distances travelled in spatial working memory. Two-way ANOVA was used to detect the effect of treatment and zone in the open-field and elevated plus-maze test, and the effect of treatment on the time spent in the familiar and novel objects of the novel object recognition test. Data from the remaining behavioral tests, body and brain weights, volumes of hippocampal layers, and total number of DCX- and NPY-immunoreactive neurons and density of VAChT-immunoreactive varicosities were analysed by one-way ANOVA, using treatment as the independent variable. Whenever appropriate, ANOVAs were followed by Tukey highest signification difference (HSD) post-hoc comparisons. Differences were considered to be statistically significant when $p < 0.05$. Since sex-related differences were not found, animals of both sexes were respectively pooled in the same group.

3. Results

3.1. Body and brain weights

Values are expressed in g (SD). On P60, mean body weights were 292.73 (13.73) for control rats, 286.94 (12.32) for ED21 rats and 274.88 (9.23) for TSHI rats, whereas mean brain weights were 1.55 (0.03) for control rats, 1.53 (0.04) for ED21 rats and 1.54 (0.03) for TSHI rats. ANOVA showed that there was no significant effect of treatment on body and brain weights.

3.2. Developmental milestones and reflex development in pups

The mean time spent by pups to turn around and move toward the upper end of the surface in negative geotaxis test is shown in Fig. 1A. Repeated measures ANOVA revealed that there was a significant main effect of treatment ($p < 0.001$), as well as a significant treatment × P day interaction ($p < 0.05$). Post-hoc tests for this interaction showed that, on P6 and P7, TSHI rats performed worse ($p < 0.05$) than controls and ED21 rats. There were no significant differences between all groups on the other days. There were no significant differences between control and ED21 rats. These results reflect normal vestibular function, motor development, and activity in ED21 rats.

The mean time spent by pups to retract from the edge is the cliff avoidance test is shown in Fig. 1B. Repeated measures ANOVA revealed that rats from all groups improved their performance during the cliff avoidance test ($p < 0.001$), and that there was a significant effect of treatment ($p < 0.001$), as well as a significant treatment × P day interaction ($p < 0.001$). Post-hoc test for this interaction showed that the TSHI rats performed worse on the cliff avoidance test during P6 and P7 ($p < 0.001$) and after that performed like the controls. The cliff avoidance reaction in ED21 rats was equivalent to that of controls, reflecting a normal processing sensorial input and well adaptive rodent behavior.

Repeated measures ANOVA applied to data from the forelimb grasp test (Fig. 1C), revealed that rats in all groups improve their performance during the test ($p < 0.001$) and that there is a significant effect of treatment ($p < 0.001$), as well as a significant treatment × P day interaction ($p < 0.05$). Post-hoc tests for this interaction showed that the TSHI rats performed worse than control and ED21 rats in the forelimb grasp test from P6 to P8 ($p < 0.05$) and that, after that, their performance was similar to that of control and ED21 rats. TSHI rats took more days to acquire forelimb grasp and their strength/resistance to fatigue was lower (although not significant) than in ED21 and control rats. There were no significant differences between control and ED21 rats. These results may result from a delay in acquisition of motor developmental milestones.

The mean forelimb strength on P10 is shown in Fig. 1D. ANOVA showed that there are no significant differences between groups in the forelimb strength. All rats had an identical proximal muscle strength and resistance to muscle fatigue.

3.3. Locomotor activity

Locomotor activity was assessed in the open field and elevated plus maze (Fig. 2). The distances, expressed in cm (SEM), travelled in the open field were 3508 (112) for control rats, 3285 (163) for ED21 rats and 3652 (86) for TSHI rats. In the elevated plus maze, these distances were 2323 (50) for control rats, 2185 (108) for ED21 rats and 2125 (154) for TSHI rats. There was no significant influence of time of birth or prenatal ischemia (TSHI) in the total distance travelled in the open field, as well as in the elevated plus maze, indicating no alterations among groups in locomotor and exploratory activity.

3.4. Anxiety

Anxiety-like behavior was evaluated in the open-field and in the elevated plus-maze (Fig. 2). In the open-field test (Fig. 2A), there was no significant influence of treatment ($p = 0.076$), but there was a significant main effect of zone ($p < 0.001$) on the distances travelled in the outer and in the inner zones of the open field. Similar to control rats, ED21 and TSHI rats travelled longer distances in the outer zone than in the inner zone of the open field. Interestingly, ANOVA revealed a

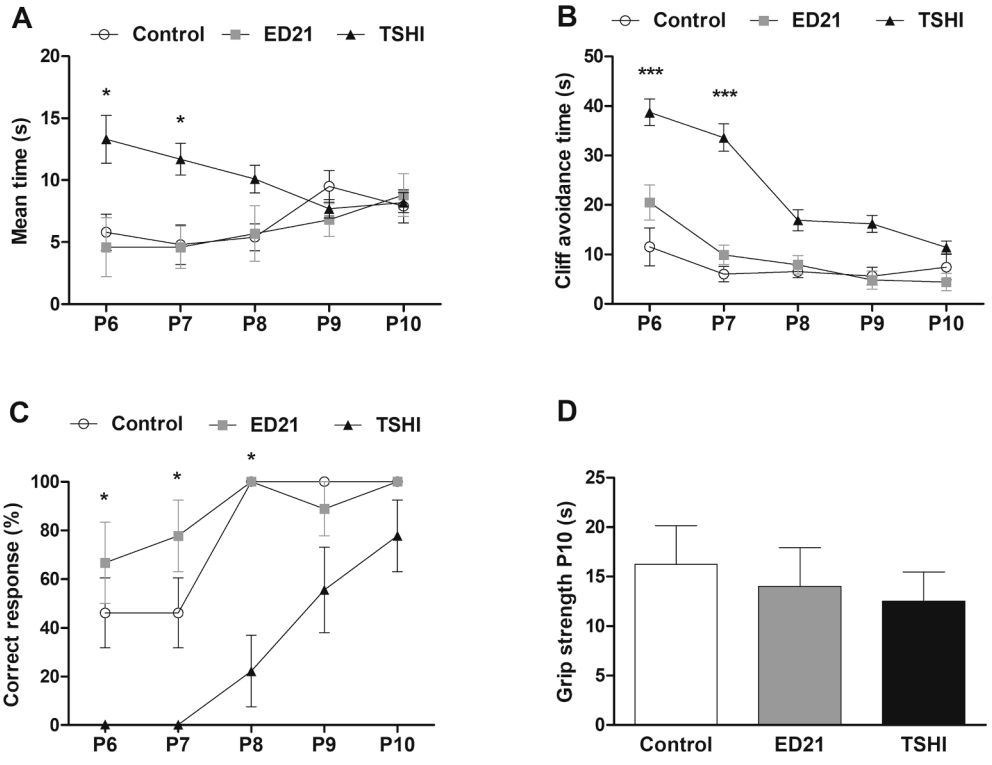

Fig. 1. Neonatal developmental milestones. (A) Negative geotaxis: based on the time required for each pup to turn and move toward the upper end of the surface. TSHI rats had a worse performance at P6 and P7 ($p < 0.05$) compared to both control and ED21 rats. (B) Cliff avoidance: based on the time required for each pup to retract from the edge of a flat surface itself. TSHI rats performed worse on the cliff avoidance test at P6 and P7 ($p < 0.001$). (C) Forelimb grasp reflex: data shown as the percentage of pups per litter that fully performed the task. TSHI rats performed worse on the forelimb grasp test at P6, P7 and P8 ($p < 0.05$). They took more days to acquire forelimb grasp. (D) Grip strength at P10: data presented based on the time pups suspend themselves by their forepaws in the metal bar. There were no significant differences between groups. All values, except percentages, are expressed as mean ± SEM. * $p < 0.05$ and *** $p < 0.001$ versus control and ED21 groups.

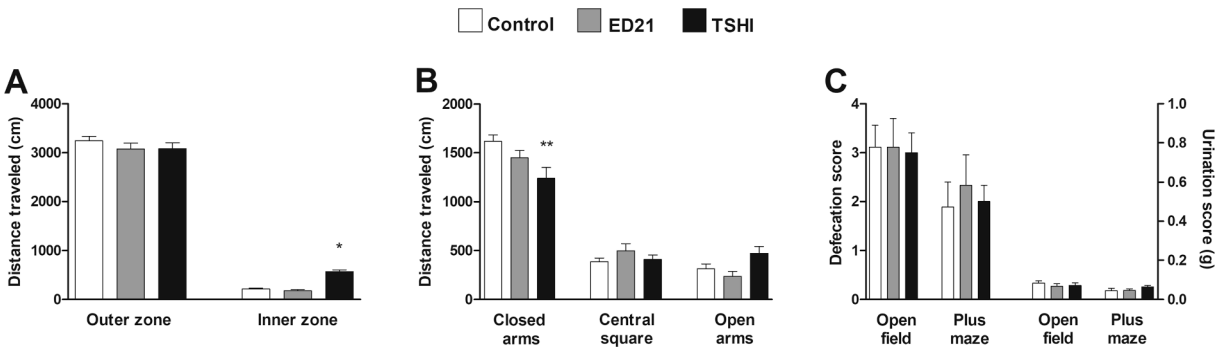

Fig. 2. Graphic representation of the distance travelled (A) in the open-field and the distance travelled (B) in the elevated plus-maze by ED21, TSHI and control rats. TSHI rats travelled significantly longer distances in the inner zone of open-field arena than control and ED21 rats. Also, TSHI rats travelled significantly lesser distances in the closed arms when compared to control but not to ED21 rats (n.s.). (C) There are no significant differences between the groups in the number of fecal boli and amount of urine deposited by rats during the open-field and elevated-plus maze tests. Data are presented as mean ± SEM. * $p < 0.05$ versus control and ED21 groups and ** $p < 0.01$ versus control group.

treatment × zone interaction ($p < 0.01$). Post-hoc analysis to this interaction showed that TSHI rats travelled significantly more distance in the inner zone of open-field arena than both control and ED21 rats ($p < 0.05$).

In the plus-maze test (Fig. 2B), there was no significant influence of treatment ($p = 0.460$), but there was a significant main effect of zone ($p < 0.001$) on the distances travelled in the different zones of the apparatus. Rats from all groups travelled longer distances in the closed arms than in the central square and opens arms of the elevated plus maze, and these differences were statistically significant. In addition, ANOVA revealed a treatment × zone interaction ($p < 0.001$) and post-hoc analysis to this interaction showed that the distance travelled by TSHI rats in the closed arms was significantly lesser than that travelled by controls ($p < 0.01$), but not by ED21 rats (n.s.). There were no significant differences between groups in the distances travelled in the open-arms and central area of plus-maze.

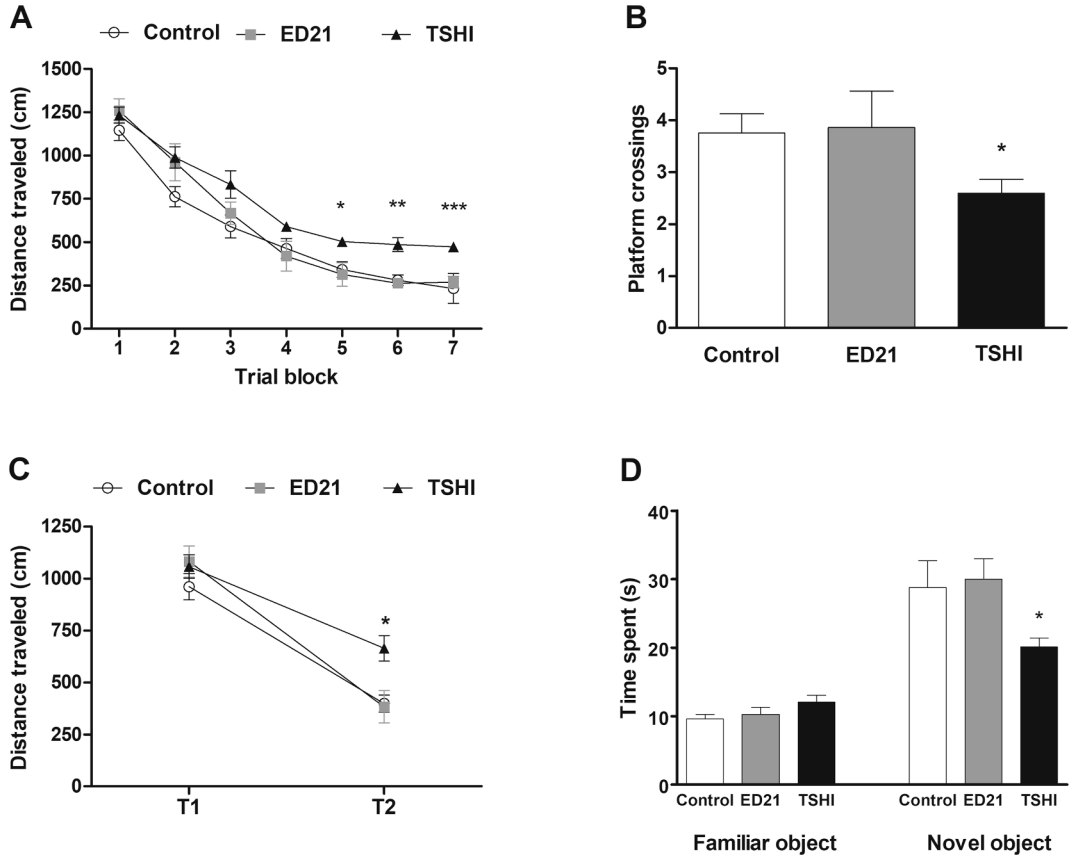

Fig. 3. Graphic representation of the distance travelled (A) and platform crossings (B) in the Morris water maze test, distance travelled on the delayed-match-to-place version of the Morris water maze test (C) and time spent in novel object recognition test (D). (A) The total distance travelled to achieve the hidden platform for each block of four consecutive trials during the acquisition task. Note that TSHI rats showed impaired performance to locate the hidden platform during acquisition compared to control and ED21 animals. (B) TSHI rats crossed the virtual position of the escape platform less frequently than control and ED21 rats. (C) On the delayed-match-to-place version of the Morris water maze to assess spatial working memory, TSHI rats showed significant worse performance in the retention trial when compared to control and ED21 rats. (D) In novel object recognition test, TSHI rats spent significant less time in the novel object when compared to control and ED21 rats. Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control and ED21 groups.

There was also no significant effect of treatment on the defecation and urination scores (Fig. 2C) in the open-field as well as in the elevated plus-maze.

3.5. Spatial learning and memory

The mean distances travelled by rats to find the submersed platform in the reference memory task of the Morris water maze are show in Fig. 3A. Repeated measures ANOVA revealed that control, ED21, and TSHI rats progressively improved their capacity to locate the hidden platform during the 14 days of acquisition ($p < 0.001$); a significant main effect of treatment in the distance travelled was also found ($p < 0.05$), as well as a significant treatment × trial blocks interaction ($p < 0.01$). Post-hoc tests showed that the performance to locate the hidden platform during acquisition was significantly worse in TSHI rats than in controls and ED21 rats in the trial blocks 5, ($p < 0.05$), 6 ($p < 0.01$) and 7 ($p < 0.001$). The analyses derived from the probe trial are shown in Fig. 3B, where it can be seen that TSHI rats crossed the virtual position of the escape platform less frequently ($p < 0.05$) than both control and ED21 rats. All rats rapidly learned to find the visible platform. The average distances swam over the 4 trials to locate the platform position, expressed in cm (SEM), were 213 (64) for controls, 231 (77) for ED21 and 228 (71) for TSHI rats. All rats had similar sensorimotor abilities as no significant differences were found when the groups were compared.

3.6. Spatial working memory

The mean distances travelled to find the platform in the information and retention trials are shown in Fig. 3C. Repeated measures ANOVA showed a significant effect of trial block ($p < 0.01$), treatment ($p < 0.05$) and treatment × trial block interaction ($p < 0.05$). Post-hoc tests revealed that TSHI rats showed significant worse performance in the retention trial than both control and ED21 rats ($p < 0.05$).

3.7. Novel object recognition

The two-way ANOVA analysis of data obtained in this test (Fig. 3D) revealed that there was a significant effect of the presence of the novel object, since animals of all groups spent more time exploring the novel than the familiar object ($p < 0.001$). Albeit no significant effect of treatment was observed, there was a significant treatment × object interaction ($p < 0.05$). Post-hoc analysis for this interaction showed that TSHI rats spent less time with the novel object when compared to both control and ED21 rats ($p < 0.05$).

3.8. Morphological data

3.8.1. DCX immunoreactive neurons

The total numbers of DCX-immunoreactive neurons in the subgranular layer of the dentate gyrus are shown in Fig. 4. One-way ANOVA revealed that there was a significant effect of treatment on the total number of DCX-immunopositive cells ($p < 0.001$). As shown by post-hoc analyses, the total number was significantly higher in TSHI rats than in control and ED21 rats ($p < 0.001$). There were no significant differences between control and ED21 rats in this parameter. The qualitative observation of the hilus of the dentate gyrus revealed the presence of ectopic DCX-immunoreactive cells in TSHI rats, but not in ED21 and control rats. We have estimated the total number of these cells and found that it was 175 ± 19 in TSHI rats.

3.8.2. NPY-immunoreactive neurons

The total numbers of NPY-immunoreactive neurons estimated in the dentate gyrus and hippocampal CA1 and CA3 subfields are shown in Fig. 5. ANOVA revealed that there was a significant effect of treatment in the total number of NPY-immunopositive cells in the hilus ($p < 0.01$) but not in CA1 and CA3 (n.s.). As shown by post-hoc analysis, the total number of NPY-immunoreactive cells was significantly higher in the dentate hilus of TSHI rats than in control and ED21 rats ($p < 0.05$). There were no significant changes in this parameter between control and ED21 rats in any of the regions analyzed.

3.8.3. Hippocampal layers volumes

The volumes of hippocampal layers are expressed in mm^3 (SD). Volumes of dentate hilus were 1.02 (0.10) for control rats, 0.96 (0.13) for ED21 rats and 0.96 (0.17) for TSHI rats; in CA3 pyramidal layer they were 1.14 (0.17) for control rats, 1.16 (0.14) for ED21 rats and 1.08 (0.14) for TSHI rats, and in CA1 pyramidal layer were 0.59 (0.07) for control rats, 0.59 (0.06) for ED21 rats and 0.56 (0.07) for TSHI rats. ANOVA showed that there was no significant effect of treatment on the volumes of the hippocampus layers.

3.8.4. VAChT-immunoreactive varicosities

The results of the areal density of VAChT-immunoreactive varicosities in the dentate hilus are shown in Fig. 6. ANOVA revealed that there was a significant effect of treatment in the number of varicosities ($p < 0.05$), and post-hoc analysis showed that this number was significantly lower in TSHI rats than in control rats ($p < 0.05$). No significant difference was found between control and ED21 rats.

4. Discussion

The main findings of the present study are that late preterm birth induced by mifepristone does not seem to induce significant brain injury or impair neurodevelopment of rats. The neonatal developmental milestones, anxiety levels, learning and memory functions postnatal neurogenesis and the hippocampal cholinergic and NPY GABAergic systems were not significantly affected by premature delivery induced by mifepristone or exposure to the extrauterine environment before term age. Conversely, prenatal hypoxia–ischemia delays neonatal developmental milestones and has deleterious consequences in learning

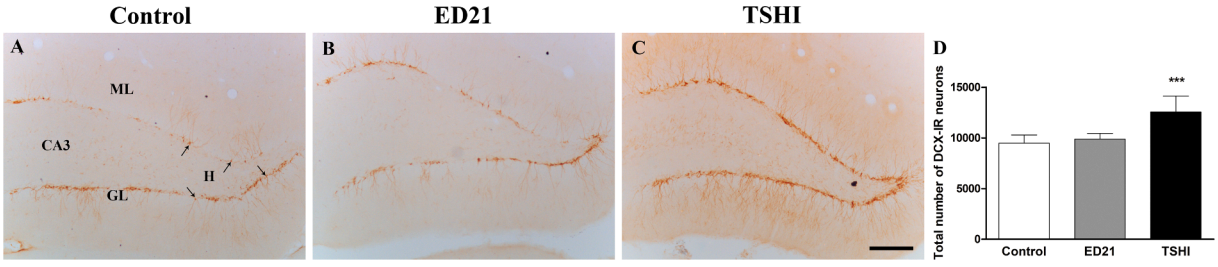

Fig. 4. Representative photomicrographs of level-matched coronal sections of the dentate gyrus immunostained for doublecortin (DCX) (A-C) and graphic representation of the total number of DCX-immunoreactive cells (D) from control, ED21 and TSHI rats. Arrows in A show DCX-immunopositive cells in the dentate gyrus subgranular layer. Note that the density of DCX cells is significantly increased in TSHI rats when compared to both control and ED21 rats. ML, dentate gyrus molecular layer; GL, granule cell layer; H, dentate hilus; CA3, pyramidal cell layer of CA3 hippocampal field. Data are presented as mean + SD. *** $p < 0.001$ versus control and ED21 groups. Scale bar = 200 μm in A-C.

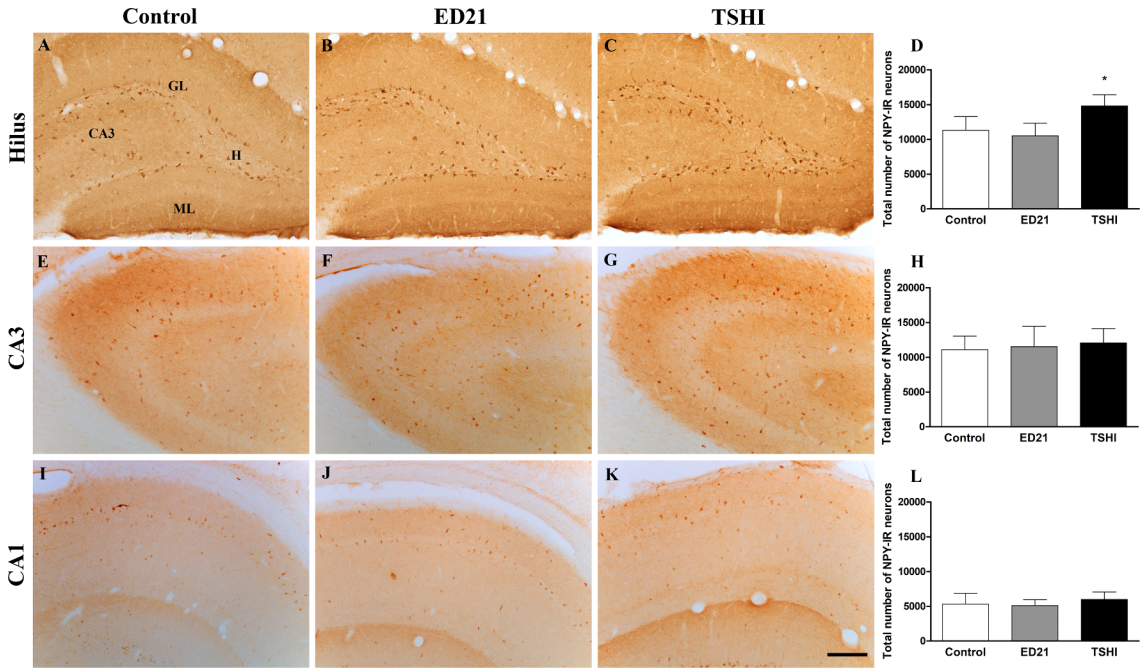

Fig. 5. Representative photomicrographs of level-matched coronal sections of the hippocampus immunostained for neuropeptide Y (NPY) (A-C, E-G and I-K) and graphic representation of the total number of NPY-immunoreactive cells in the dentate hilus (D) and CA3 (H) and CA1 (L) hippocampal regions from control, ED21 and TSHI rats. The density is significantly increased in the hilus of TSHI rats (C), but not in CA3 and CA1 regions. ML, dentate gyrus molecular layer; GL, granule cell layer; H, dentate hilus; CA3, pyramidal cell layer of CA3 hippocampal field; CA1, pyramidal cell layer of CA1 hippocampal field. Data are presented as mean + SD. * $p < 0.05$ versus control and ED21 groups. Scale bar = 200 μm in A-C, E-G and I-K.

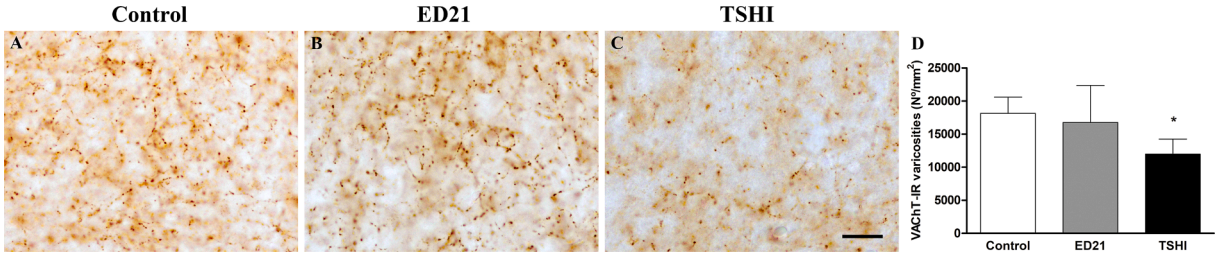

Fig. 6. Representative photomicrographs of level-matched coronal sections of the dentate hilus immunostained for vesicular acetylcholine transporter (VAChT) (A-C) and graphic representation of the areal density of VAChT-immunoreactive varicosities (D) of control, ED21 and TSHI rats. The density of varicosities is significant smaller in TSHI rats than in control and ED21 rats. Data are presented as mean + SD. * $p < 0.05$ versus control group. Scale bar = 20 μm in A-C.

and memory process as well as in neurogenesis, cholinergic innervation and NPY expression in the hippocampus.

In literature, most of the rodent models that have been used to evaluate the encephalopathy of prematurity correspond to injury-induced models, with triggers such as ischemia, hypoxia or infection (McCarthy et al., 2018). Additionally, because many of these models do not result in premature delivery, we sought after a non-injury animal model that would reveal the consequences to the brain of premature delivery and not the effects of insults during perinatal period. We used mifepristone, that induced premature birth at ED21, and we found that it does not affect neonatal neurologic development and does not impact locomotion and exploratory activity, learning and memory or anxiety, demonstrating normal functioning of the different components of nervous system tested in this work. Furthermore, mifepristone-induced preterm birth did not lead to alterations in postnatal neurogenesis or in the cholinergic and NPY GABAergic systems of the hippocampal formation. With this approach, we showed that late preterm birth rat, in a non-inflammatory environment, does not seem to constitute a handicap for neurologic development. Only in a model with an external insult, i.e., the TSHI model, we observed neurologic impairment. By analogy, we speculate that neurologic impairment observed in preterm human infants may arise from exposure to environmental insults during the perinatal period (as demonstrated in the TSHI inflammatory model) and not because of premature delivery or premature exposure to extrauterine environment. In humans, this hypothesis is supported by the fact that preterm newborns with hypoxia–ischemia related risk factors (acidemia, low Apgar scores, apneia, respiratory distress syndrome and seizures) are at increased risk of periventricular leukomalacia, the hallmark of encephalopathy of prematurity (Huang et al., 2017). Similarly, preterm newborns exposed to different types of triggers inducing inflammation (infection, hypotension, pain barotrauma, volutrauma, stress) in neonatal intensive care units (Martens et al., 2003; Miall-Allen, de Vries, & Whitelaw, 1987; Penn, Gressens, Fleiss, Back, & Gallo, 2016) can develop encephalopathy of prematurity (Back & Miller, 2014). This hypothesis also matches with the progressive better neurologic outcome of preterm infants that has been observed in the last decades, in parallel with improved care in neonatal units (Hack & Costello, 2008).

Our data agrees with the earlier observation made by Burd and collaborators (Burd et al., 2010) that prenatal administration of mifepristone does not alter neuronal morphology and the number of dendrites in primary neuronal cultures from the mouse foetal cortex, as

opposed to what happens after intrauterine administration of lipopolysaccharide (Burd et al., 2010). This suggests that only inflammation, and not the time of delivery, results in changes in neuronal morphology. Interestingly, our results extend the findings of Burd and collaborators (Burd et al., 2010), which were focused on foetal neurology, and are pioneering in showing that mifepristone-induced preterm birth does not significantly impact postnatal neurodevelopment nor anxiety and cognitive functions evaluated in adolescent rats. Moreover, by showing the absence of changes in postnatal neurogenesis, hippocampal NPY GABAergic and cholinergic systems our results do not support the concept of dysmaturation of the nervous system induced with prematurity but, instead, corroborate the view of a benign course of maturation of the nervous system in a non-inflammatory environment. It deserves to be mentioned here that in a recent study using mifepristone to induce preterm birth in mice (Cho, Hong, Hong, Oh, & Kim, 2015), it was shown that, despite the increased expression of IL-1β pro-inflammatory cytokine, no changes were found in the neuronal marker NeuN, which suggests that albeit mifepristone might have an acute effect in inflammation it does not affect neuronal viability.

To better understand the potential alterations caused by the non-inflammatory, mifepristone-induced, model of preterm birth we decided to study in parallel an inflammatory hypoxia–ischemia model of preterm brain injury. Post-natal hypoxia–ischemia models are well established models for preterm (namely the neonatal hypoxia–ischemia induced in P3), and there is valuable information about the molecular, histological and behavioral outcomes in these models.(Hagberg, Peebles, & Mallard, 2002; Sanches, Arteni, Nicola, Aristimunha, & Netto, 2015). Because postnatal ischemic models of encephalopathy of prematurity are well known and lack the maternal, placental, and foetal components that may be important to replicate full preterm brain injury, we choose a prenatal hypoxia–ischemia model that, when applied to rats at ED17 or ED18, can affect pre-oligodendrocytes and reproduce the white matter injury found in preterm newborns (Jantzie & Robinson, 2015b). Hypoxia-ischemia insults in rodents between ED17 and ED18 appear to correspond to intrauterine events occurring in human infants at 23–25 weeks of gestation (Jantzie & Robinson, 2015b). While most studies of prenatal ischemia have been used to elucidate cellular and molecular mechanisms and to develop therapeutic strategies, in the present work we used this model to evaluate the neurodevelopment trajectory in pups and the effects on emotions and cognition during adolescence. Our results show that TSHI rats have delayed neurodevelopment and significant changes in learning and memory functions and anxiety as well as in postnatal neurogenesis and in the hippocampal cholinergic and NPY GABAergic systems. In agreement with earlier observations made by Lubics and collaborators (Lubics et al., 2005), we found that TSHI rats took more time to develop the normal response in cliff avoidance, negative geotaxis and forelimb grasp, which indicates a delay in the acquisition of normal reflexes. The behavioral studies performed in rats between 30 and 60 days of age, showed that ED21 and TSHI rats exhibit normal locomotor activity, which indicates that none of the models used in the present study affects, at least significantly, the neural structures implicated in the complex system that regulates locomotor behavior. Data obtained in the open-field and elevated plus-maze also showed that ED21 and control rats experienced normal anxiety levels while TSHI rats exhibited decreased anxiety levels. In effect, TSHI rats engaged in risky behavior in the elevated plus maze test and open field, as they spent more time exploring the exposed areas and remained less time in the protected areas than ED21 and control rats. This finding is in keeping with data obtained in a previous work (Ming-Yan et al., 2012) showing that, after prenatal hypoxia–ischemia, rats show reduced anxiety, fear and inhibition of the risky behavior, but is at odds with data from Sab and collaborators (Sab et al., 2013) who showed that adult rats that had been exposed to hypoxia–ischemia on ED18 exhibited increased anxiety in the plus-maze test, but not in the open-field test. It is known, from studies in humans, that infants who suffer perinatal brain injury, including those with encephalopathy of prematurity, are prone to anxiety and impulsivity (Réveillon, Borradori Tolsa, Monnier, Hüppi, & Barisnikov, 2016; Salmaso, Tomasi, & Vaccarino, 2014). The balance between anxiety and impulsivity could be a possible explanation for the discrepant results detected in animal studies. Time spent in the central area of the plus-maze has been also correlated with impulsivity, and this characteristic could similarly explain the increased exploratory activity observed in TSHI rats (Leonardo Rico, Hurtado-Parrado, Vásquez-Sepúlveda, Fonseca, & Cardona, 2016). Interestingly, Delcour and collaborators (Delcour et al., 2012) reported that prenatal hypoxia–ischemia induces increased exploration and activity in adult rats. Like these authors, we have also found increased exploration levels in TSHI rats, but without significant changes in general locomotor activity. This apparent discrepancy is explained by the fact that the increased exploration shown by TSHI rats only occurred in the exposed areas, both of open-field and plus maze, but not in the closed arms of plus-maze where the distance travelled by TSHI was reduced.

In contrast to ED21 rats who showed normal spatial learning and memory functions, TSHI rats performed worse than ED21 and control rats during the acquisition phase of the spatial version of Morris water maze, particularly in the last three trial blocks. This impairment in learning acquisition had consequences in the spatial memory evaluated in the probe trial, demonstrated by lesser frequency that TSHI rats crossed the former position of the platform. It is important to note that in a previous study that used a similar model, no alterations in spatial learning and memory were found in adult rats, despite the presence of deficits in short- and long-term object memory tasks (Delcour et al., 2012). In other study it was shown that a 30-min prenatal hypoxia–ischemia insult on ED17 impaired spatial learning, but not the retention of spatial memory, in rats aged 28 days (Cai, Xiao, Lee, Paul, & Rhodes, 1999). The finding in our study that a 60-min hypoxia–ischemia insult induced on ED18 impaired not only spatial learning, but also spatial memory indicates that the duration of the hypoxia–ischemia insult is, very likely, a determining factor of the severity of the neurological consequences. These results reinforce the view that even the late prenatal phase is a period of great vulnerability of the neural substrate that mediates hippocampal-dependent learning and memory to the hypoxia–ischemia insults.

Data obtained in the delayed-match-to-place version of the Morris water maze and in the new object recognition test, where rats were tested to discriminate a new object from a familiar object to which they had been exposed 2 h before, showed that short-term memory was unaltered in ED21 rats, as opposed to what happened in TSHI rats. Specifically, TSHI rats exhibited less interest to novelty than ED21 and control rats, as shown by the reduced time they spent with the novel object, when compared to the time spent with the familiar object, in the recognition test. This behavior of TSHI rats might result from impairments in encoding object features or disturbances in the ability to retrieve (short-term memory) and discriminate old from new information. Nevertheless, the influence of anxiety and attention in the behavior observed in TSHI rats cannot be overlooked. Fails in object recognition may result from attention deficits and, when associated with increased impulsivity/less anxiety observed in the open-field test, recapitulate some of the symptoms of the attention deficit hyperactivity disorder commonly found in preterm children. This situation has already been addressed by Delcour and collaborators (Delcour et al., 2012) in their model of prenatal ischemia, induced by unilateral ligation of uterine artery at ED17.

Interestingly, in the present work we have also found that ED21 rats had no alterations of postnatal neurogenesis in the dentate gyrus. It is known that preterm infants are frequently exposed prenatally to glucocorticoids (Roberts, Brown, Medley, & Dalziel, 2017), and that glucocorticoids receptors are present at high levels in the hippocampus and are involved in dexamethasone-induced apoptosis (Amaral, Solá, Steer, & Rodrigues, 2009). In the neonatal period, exposure to dexamethasone results in marked apoptosis among the progenitor cells in the dentate gyrus (Yu et al., 2010). Mifepristone is an antiprogesterone and

antiglucocorticoid synthetic steroid that, in rat pups, is able to reduce dexamethasone-induced apoptosis in neuroglial progenitor cells (Sze et al., 2013). It is believed that this action is partially mediated by its capacity to regulate corticosteroid receptor expression in dentate gyrus (Llorens-Martín et al., 2011). However, it is not known if, in the absence of inflammation or increased steroid levels, mifepristone can interfere with neurogenesis. Also, the intrinsic antioxidant properties of mifepristone are thought to promote neuronal survival, which could ameliorate the outcome of preterm newborns with an extra pool of progenitor cells (Behl et al., 1997). Previous works have paid attention to the effects of mifepristone in preterm birth, but its effects on neurogenesis were not elucidated. Our finding of unchanged postnatal neurogenesis in ED21 rats, lends support to the proposal that the effects of mifepristone are particularly potent in environments with high glucocorticoid levels and that it does not modify proliferation or survival in control animals (Oomen, Mayer, De Kloet, Joëls, & Lucassen, 2007). It is important to note, however, that it cannot be inferred from our data if the absence of alterations of neurogenesis in ED21 rats means that preterm birth does not induce changes in neurogenesis by itself or if mifepristone induces direct neuroprotection. In contrast to ED21 rats, TSHI rats had an increased number of DCX cells in the subgranular zone of the dentate gyrus without changes in the layer volume, which indicates that the prenatal hypoxia/ischemia model used in this study increases postnatal neurogenesis. Identical observations were done by other authors that used experimental models of neonatal hypoxic-ischemic brain injury (Brégère et al., 2017) and intermittent post-natal hypoxia (Bouslama et al., 2015). In addition to the increased number of DCX in the subgranular zone of the dentate gyrus, we have also found aberrant DCX cells in the hilus of the dentate gyrus of TSHI rats. This finding points to a proactive response to injury with increased cellular response of progenitor cells, but with an imperfect repair (dysmaturation). Because postnatal neurogenesis in this region is believed to contribute to learning and memory function (Alam et al., 2018; Deng, Aimone, & Gage, 2010), it is possible that the impaired spatial learning and memory observed in TSHI rats might be explained, in part at least, by the altered neurogenesis and aberrant migration of new neurons in the dentate gyrus. Overall, these findings provide a new mechanism by which exposure to prenatal ischemia may lead to long-term memory adverse outcomes and are in accordance with recent literature reporting the importance of adult neurogenesis in consolidating memories during sleep in mice (Kumar et al., 2020). In contrast to ischemic models, intrauterine infection/lipopolysaccharide models of preterm birth have been reported to significantly reduce hippocampal neurogenesis and, like in our work, to be associated with ectopic location of granule cells (Hester, Tulina, Brown, Barila, & Elovitz, 2018), which indicates that the exposure to intrauterine inflammation disrupts early postnatal neurogenesis and leads to aberrant migration of newly born granule cells. The result of ischemia and infection models are the same, as they both lead to a significant postnatal neurologic impairment, but the pathophysiologic mechanisms involved in each model are probably different.

Comparing the two models used in the present study, the fact that we have found no alterations in learning and memory associated with unaffected neurogenesis in ED21 and control rats supports the idea that disruption of neurogenesis is possibly one mechanism involved in the learning and memory deficits recognized in preterm children exposed to ischemic insults.

Studies using autopsy samples of preterm human infants have shown relatively few numbers of NPY-positive neurons in the cortex (Panda et al., 2018). Also, studies in rodents have shown that neonatal hypoxia–ischemia leads to a significant reduction in the number of NPY-positive neurons in the hypothalamus, amygdala and stria terminalis (Carty et al., 2010). In the present study, the estimation of the total number of NPY-immunoreactive neurons revealed that the non-inflammatory, mifepristone-induced preterm birth does not alter the expression of NPY in the hilus of the dentate gyrus and in the CA1 and CA hippocampal subfields. However, in TSHI rats the total number of NPY neurons was significantly increased in the hilus of the dentate gyrus without changes in layer volume. This finding is particularly interesting because, given the neurotrophic and neuroprotective effects of NPY (Gøtzsche & Woldbye, 2016; Xapelli et al., 2008), such increase could denote a NPY-dependent pathway by which ischemia would lead to increased neurogenesis - Y1 receptor-mediated neurogenesis (Andersen & Gundersen, 1999), or represent only a marker of neurogenesis *per se* (Gøtzsche & Woldbye, 2016). NPY has an important role in neuroplasticity, neurotransmission, and memory(Gøtzsche & Woldbye, 2016; Michaelson et al., 2020). Spatial memory training leads to increased hippocampal NPY gene expression that, together with NPY-mediated neurogenesis, could be important in consolidation and long-term retention of spatial memory (Gøtzsche & Woldbye, 2016). In our work, TSHI rats have done spatial memory training and, despite having increased numbers of NPY neurons in the dentate hilus, they had impaired spatial memory tests when compared to controls. This suggests that the increase in the number of hilar NPY neurons, was not enough to compensate the lesion induced by prenatal hypoxia–ischemia. It is also known that NPY has anticonvulsive properties and that the expression of NPY in the hippocampus increases in response to enhanced neuroexcitability and alterations of the local circuitry (Bacci, Huguenard, & Prince, 2002; Cardoso, Freitas-da-Costa, Carvalho, & Lukoyanov, 2010). Interestingly, it was recently shown that term neonates with seizures have higher CSF levels of NPY (Tanrıverdi et al., 2020). Once more, comparing the present two models, in the mifepristone-induced preterm birth there were no alterations in NPY levels, suggesting that, conversely to the preterm hypoxia–ischemia model, there was no massive alteration of the hippocampus neuronal circuitry and neuro-excitability. Overall, NPY neurons may be important players in injury repair and their role should be further explored.

It is known that hilar GABAergic interneurons, particularly those expressing NPY, are main targets for the septo-hippocampal cholinergic projections and contain a variety of cholinergic receptors (Dougherty & Milner, 1999; Feduccia, Chatterjee, & Bartlett, 2012). Knowing this and assuming that the NPY-ergic system may be interdependent of the cholinergic innervation in the cerebral cortex (Cardoso, Silva, Magano, Pereira, & Andrade, 2014; Milner, Wiley, Kurucz, Prince, & Pierce, 1997), we also sought to analyze the effects of prematurity and prenatal hypoxia–ischemia in the cholinergic system of the hippocampus. Our results show that the density of VAChT-immunoreactive varicosities does not differ between ED21 and control rats, advocating that preterm birth does not induce enough alterations, locally and in the basal forebrain, to provoke changes in the cholinergic innervation of the dentate gyrus. In contrast, we have found that the density of the cholinergic varicosities was significantly reduced in the dentate hilus of TSHI rats. Since we did not find changes in the volume of dentate hilus in TSHI rats, the reduction of the areal density of cholinergic varicosities it is not related to loss of volume of the hilus but, actually, loss of varicosities. To the best of our knowledge, studies that have analysed the effects of prematurity in the brain cholinergic system are scarce. However, in one study performed in humans it was shown that in adults born prematurely the volume of the basal forebrain cholinergic nuclei was reduced (Grothe et al., 2017). It is therefore tempting to assume that the reduction in the density of cholinergic varicosities that we have found in the dentate gyrus of TSHI rats might reflect dysfunction of the cholinergic basal forebrain, since these nuclei give rise to the majority of cholinergic projections to the hippocampus (Mesulam, Mufson, Wainer, & Levey, 1983; Woolf & Butcher, 2011). Taking into account the established relationship between preterm birth, reduction of cholinergic basal forebrain volume and cognitive impairment in adults (Grothe et al., 2017), it is also plausible to argue that the cognitive impairment observed in the TSHI rats could be explained, in part at least, by the reduction in the cholinergic innervation of the hippocampal formation.

One limitation of our work is the grade of prematurity of the rats studied. While using a rat model of preterm brain injury, we are aware

about its limitations. Rat has a lisencephalic brain, and its central nervous system at birth is more immature compared to human central nervous system, has a less prominent subplate, a greater gray/white matter ratio and a clinical course of illness different from human preterm infants. We used preterm rats delivered only two days before term. Although they represent very premature brain when compared with human brain development, we had some cautions in our conclusions because, regarding gestation duration, it represents a mild form premature delivery. Twenty-one days of gestation represent 90% of gestation length in rats. In a straightforward comparison to humans, 90% of the length of gestation in humans corresponds to 36 weeks of gestation. In this perspective, our preterm rats represent late preterm humans. However, in terms of brain development, ED21 rats represent very premature human brains of approximately 24 weeks of gestation, i.e., in the limit of human survival. From this brain development viewpoint, we studied a rodent model representative of the most preterm humans. The model we select to mimic prenatal inflammation and produce brain injury included hypoxia and ischemia. In last years, there is a great discussion about ischemia and hypoxia as determinants of brain injury in preterm infants (Gilles, Gressens, Dammann, & Leviton, 2018), with some authors indicating that the evidence for ischemia and hypoxia is low. Even considering that in the majority of preterm children hypoxia and ischemia are not a main contributor to brain damage, it is undeniable that hypoxia and ischemia are, at least in part, responsible for brain injury in some preterm infants (Laptook, 2016). There is a growing amount of literature about hypoxic-ischemic encephalopathy in preterm newborns and some authors have discussed about the efficacy of hypothermia in preterm newborns (Herrera et al., 2018; Rao et al., 2017). Indeed, it is much more difficult to detect ischemia in a preterm when compared with a term infant, and probably that is one of the reasons to be underdiagnosed. Even with the uncertainly of the contribution of ischemia, is important to continue exploring this model. Our work allowed us to uncover some links between pathophysiology of ischemia and clinical deficits, one of the best ways to choose therapeutic targets.

Another issue that it is important to discuss is the sexual dimorphism. It is well known that there is an effect of sex following the Rice-Vanucci model of neonatal hypoxia–ischemia (Netto, Sanches, Odorcyk, Duran-Carabali, & Weis, 2017; Rosenkrantz, Hussain, & Fitch, 2019). However, in the present study we did not find any significant effect of sex, both in behavioral and morphological analysis. In the present study, we used a prenatal hypoxia–ischemia model and not the Rice-Vanucci model, and consequently the changes induced by our model may, indeed, not be influenced by sex. Actually, even in prenatal hypoxia–ischemia models there are studies that found sex differences in some behavioral tests (Hermans, Hunter, McGivern, Cain, & Longo, 1992; Tashima, Nakata, Anno, Sugino, & Kato, 2001), but not in others (McCullough & Blackman, 1976; Piešová et al., 2020; Tashima et al., 2001). Albeit the discrepant studies, most of the literature seems to suggest that there is sex dimorphism in the susceptibility to hypoxia–ischemia, at least in some of the behavioral studies, but in the present study we were not able to detect significant sex dimorphism. This could be justified by the number of animals, i.e., when divided per group and per sex the relative low number of animals used in the present study may, indeed, not be enough to detect a potential sex dimorphism in the different treatments, mostly because female rats were analysed at random phases of the estrous cycle. Nevertheless, this is a very important issue that needs to be better understood, given the great importance of neonatal sex dimorphism as part of treatment strategies used in infants.

In conclusion, this is the first study in which the neurologic outcome of rats born after prenatal administration of mifepristone was assessed prospectively. Our results show that the exposure to the extrauterine environment before term age in the absence of inflammation does not seem to affect the neurologic outcome. Conversely, prenatal hypoxia–ischemia delays neonatal developmental milestones and impairs working and long-term memory and learning as well hippocampal postnatal neurogenesis, the cholinergic innervation and NPY expression in hippocampus. Prenatal ischemia alters neurogenesis, eventually by an NPY-dependent pathway, and this could explain part of long-term memory deficits. Reduction in the density of cholinergic varicosities we have found in the dentate gyrus of TSHI rats might reflect dysfunction of the cholinergic basal forebrain and constitute one possible explanation for cognitive deficits in children exposed to prenatal hypoxia–ischemia. In future works mifepristone-induced preterm birth could be combined with hypoxia–ischemia or other type of injury in an attempt to recapitulate the full spectrum of preterm brain injury in humans.

CRediT authorship contribution statement

Ruben Rocha: Conceptualization, Methodology, Investigation, Writing - original draft. **Leonardo Andrade:** Visualization, Investigation. **Tânia Alves:** Investigation. **Susana Sá:** Investigation. **Pedro A. Pereira:** Investigation. **M. Dulce Madeira:** Supervision. **Armando Cardoso:** Investigation, Formal analysis, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Repurposed Edaravone, Metformin, and Perampanel as a Potential Treatment for Hypoxia–Ischemia Encephalopathy: An In Vitro Study

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Abstract: Hypoxia–ischemia encephalopathy results from the interruption of oxygen delivery and blood flow to the brain. In the developing brain, it can lead to a brain injury, which is associated with high mortality rates and comorbidities. The hippocampus is one of the brain regions that may be affected by hypoxia–ischemia with consequences on cognition. Unfortunately, clinically approved therapeutics are still scarce and limited. Therefore, in this study, we aimed to test three repurposed drugs with good pharmacological properties to evaluate if they can revert, or at least attenuate, the deleterious effects of hypoxia–ischemia in an in vitro model. Edaravone, perampanel, and metformin are used for the treatment of stroke and amyotrophic lateral sclerosis, some forms of epileptic status, and diabetes type 2, respectively. Through cell viability assays, morphology analysis, and detection of reactive oxygen species (ROS) production, in two different cell lines (HT-22 and SH-SY5Y), we found that edaravone and low concentrations of perampanel are able to attenuate cell damage induced by hypoxia and oxygen-glucose deprivation. Metformin did not attenuate hypoxic-induced events, at least in the initial phase. Among these repurposed drugs, edaravone emerged as the most efficient in the attenuation of events induced by hypoxia–ischemia, and the safest, since it did not exhibit significant cytotoxicity, even in high concentrations, and induced a decrease in ROS. Our results also reinforce the view that ROS and overexcitation play an important role in the pathophysiology of hypoxia–ischemia brain injury.

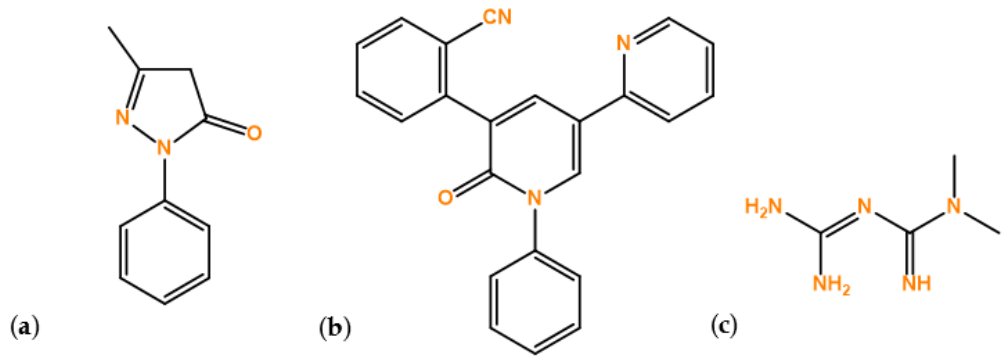
Keywords: hypoxia–ischemia; edaravone; perampanel; metformin; OGD; ROS; overexcitation

1. Introduction

Oxygen and glucose are both essential for normal brain activity. Hypoxia–ischemia results from the combination of a lack of oxygen and blood flow interruption, and, during development, it can lead to brain injury, clinically manifested as encephalopathy and seizures. These conditions are very prevalent in neonates, particularly preterm ones, being the leading cause of neonatal deaths. They are also commonly associated with important neurological disabilities in those that survive [1,2].

Brain injury due to hypoxia–ischemia is an evolving process that can be divided into three periods [3]. The first one starts immediately after the insult, lasts approximately 6 h, and corresponds to the primary energy failure phase. In this phase, due to the decreased supply of oxygen and glucose, cells switch to anaerobic metabolism, increasing lactate production and decreasing adenosine triphosphate (ATP) production [4]. Consequently, membrane ion pumps start to fail and lead to sodium and calcium accumulation within the cells, thus releasing, from degrading structures, excitatory neurotransmitters, such as glutamate [3,5]. Glutamate is a neurotransmitter that stimulates different neuro-glial receptors, namely N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. When in excessive amounts, it promotes a cascade response that eventually leads to excitotoxicity and reactive oxygen and nitrogen species production [6]. In the secondary energy failure phase (6 to 72 h after the insult), reperfusion is restored, which can intensify cell injury despite limiting brain damage. In the meantime, excitotoxic neurotransmitters and free radicals continue to be released, and reactive oxygen species (ROS), as well as other reactive species, are produced, thus leading to aggravation of mitochondrial dysfunction, cell death, and induction of brain self-inflammatory response [4,7]. Eventually, lesions begin to heal or settle into a chronic inflammation phase. Different mechanisms may occur during this tertiary phase (lasting months to years), including epigenetics and inflammatory changes that may induce cell death, repair, or remodeling [2,8].

Overall, neonatal hypoxia–ischemia brain injury evolves over time, which creates a window of treatment opportunity. There are different treatment approaches targeting the critical times of the different phases. Namely, for the first phase, it makes sense to try neuroprotective glutamate receptor blockers and free radical scavenger drugs [3]. After the initial phase, it is sensible to focus on anti-inflammatory, neuroprotective, and nerve regenerating drugs in order to restrain injury and promote healing. However, at present, therapeutic interventions that are clinically approved for the treatment of neonatal hypoxia–ischemia brain injury are still very limited [1]. Using repurposed drugs is potentially one way to progress faster and achieve efficient treatments. Among the repurposed drugs, we selected edaravone, perampanel, and metformin (Scheme 1).



Scheme 1. Structure of the repurposed drugs used in this study: (**a**) Edaravone, (**b**) Perampanel, and (**c**) Metformin.

Edaravone is a free radical scavenger [9], already approved for the treatment of amyotrophic lateral sclerosis and stroke [10]. It plays an important role not only in quenching free radicals and inhibiting lipid peroxidation [11], but also in exerting protective effects, such as anti-oxidative and pro-inflammatory responses [12].

Perampanel is an antagonist of the AMPA glutamate receptors and, therefore, is involved in inhibiting neuronal overexcitation, which may lead to neuronal protection. It has shown positive effects on seizure control in children and adults [13,14].

Metformin has been widely used to treat diabetes type 2 and is being investigated for the treatment of metabolic syndrome [15]. Its primary pharmacological activities (antioxidant and anti-inflammatory properties) can be mainly mediated by the activation of

AMP-activated protein kinase (AMPK), which subsequently may modulate oxidative stress, prevent mitochondrial damage, and enhance angiogenesis [16,17]. Overall, metformin is potentially neuroprotective.

Due to their properties, these drugs have been intensely investigated for preventing neuronal damage and treating brain disorders. For instance, edaravone has already been studied for its potential to decrease neuronal deficits consequent to traumatic brain injury [18], cerebral ischemia [10,19,20], and even in a hypoxia–ischemia model [21]. Metformin also has been vastly investigated in stroke, ischemia, and dementia [17]. However, until now, there are insufficient studies that have focused on the effects of these drugs and their effects on hypoxia–ischemia brain injury.

Hence, in this work, we aimed to repurpose edaravone, perampanel, and metformin drugs in a cell-induced hypoxia–ischemia model. Using cell viability as well as ROS production, we first sought to focus on these drugs' effects on two different cell lines, hippocampal HT-22 cells and neuroblastoma cells. Secondly, we wanted to test their potential beneficial effects on two different models of hypoxia, one using only a hypoxic atmosphere and the other using oxygen–glucose deprivation. Drugs were tested in several concentrations and times of exposure.

2. Materials and Methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin mixture were obtained from Millipore Sigma (Merck KGaA, Darmstadt, Germany). Dulbecco's Modified Eagle's Medium with no glucose (cat. no. 11966025) was obtained from Gibco (Thermo Fisher Scientific, Inc, Waltham, MA, USA). Thiazolyl blue tetrazolium bromide (MTT; cat. no. M5655), neutral red solution (NR; cat. no. N2889), sulforhodamine B (SRB; cat. no. S1402), 2, 7- dichlorofluorescein diacetate (DCFH-DA; cat. no. D6883), edaravone (cat. no. M70800), and metformin (cat. no. 317240) were obtained from Sigma–Aldrich (Merck, Algés, Portugal). Perampanel (cat. no. 23003) was obtained from Cayman (Tallinn, Estonia).

2.2. Cell lines and Cell Culture

The immortalized mouse hippocampal HT-22 cell line was kindly offered by Mitochondria and Neurodegenerative Disorders, CNC group. Human SH-SY5Y neuroblastoma cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Both cell lines were maintained, according to recommendations, at 37 °C and 5% CO_2, in DMEM medium supplemented with 10% FBS and 1% of an antibiotic mixture (penicillin and streptomycin). The medium was changed at least twice a week and trypsinized once a week, and it was only made when cells reached 80% or more confluence. Cells were seeded, in 96-well plates, at a density of 1×10^4 cells/mL for HT-22 cells and at 1×10^5 cells/mL for SH-SY5Y cells. After seeding, cells were allowed to adhere for 24 h, before exposure to the different treatments.

2.3. Cells Treatment

Edaravone and perampanel were dissolved in DMSO (0.1% in cell culture medium) and metformin in sterilized water (1% in cell culture medium). The three drugs were tested in cells with concentrations ranging from 0.1 to 100 μM. All treatments were performed for 6, 24, 48, and 72 h after cell attachment. For each drug, control wells were added with 0.1% of DMSO or 1% of sterilized water.

2.4. Oxygen Glucose Deprivation and Hypoxia Models

In oxygen–glucose deprivation (OGD) and hypoxia experiments, twin plates were created, and the induction model was performed as previously described [22,23]. For hypoxia, one twin plate was placed in a hypoxia incubator chamber (StemCell cat no.27310), with a 2% O_2, 10% CO_2, and 88% N_2 atmosphere, while the other twin plate was placed

in normoxia (21% O_2). Similarly, in OGD experiments, one twin plate was placed in the hypoxia incubator chamber, while the other twin plate was placed in normoxia. However, in this model, the plate submitted to hypoxia was cultured in DMEM-free glucose medium.

2.5. Cell Morphology Assessments

Cell morphology was assessed using a Leica DMI6000 B automated microscope (Wetzlar, Germany). After all treatments, cell morphology was examined and images were captured using Leica LAS X imaging software v3.7.4 (Leica Microsystems, Wetzlar, Germany).

2.6. Cell Viability Assays

At the end of the incubation periods, cellular viability was assessed using MTT, SRB, and NR assays. For the MTT assay, the cell medium was removed, and 100 µL of MTT solution (0.5 mg/mL in PBS) was added to each well, followed by 3 h of incubation protected from light. Then, the solution was aspirated and DMSO (100 µL/well) was added to solubilize formazan crystals. Absorbance was then measured at 570 nm, using an automated microplate reader (Tecan Infinite M200, Tecan Group Ltd., Männedorf, Switzerland). To perform the SRB protocol, the cell medium was removed, and cells were firstly washed with PBS solution. After that, cells were fixed with 10% cold trichloroacetic acid for 30 min and subsequently stained, protected from light, with 200 µL/well of SRB for 1 h. Afterwards, the plate was washed with tap water several times to remove excess dye. Finally, the dye bound to the proteins was dissolved with Tris-NaOH solution (10 mM). Absorbance was measured using a microplate reader with a wavelength of 540 nm (Tecan Infinite M200, Tecan Group Ltd., Männedorf, Switzerland). For the NR assay, 100 µL of the NR medium (1:100 in culture medium) was added to each well after the cell medium removal. After 3 h of incubation, cells were washed with 150 µL of PBS, and 150 µL of NR destain solution was added to each well. Afterward, absorbance at 540 nm was measured by using the microplate reader (Tecan Infinite M200, Tecan Group Ltd., Männedorf, Switzerland).

2.7. ROS Evaluation

Intracellular ROS production was measured using the fluorescent dye, DCFH-DA. Prior to drug exposure, cells were incubated for 30 min with 100 µL of DCFH-DA diluted in 1000× culture media. At the incubation end, the solution was removed, and cells were incubated with the respective drugs. Then, fluorescence was detected after the 6, 24, and 48 h treatment periods using a fluorescence plate reader (SpectraMax Gemini EM Microplate Reader, Molecular Devices, San Jose, California), with filters at 485 nm excitation and 530 nm emission.

2.8. Statistical and Data Analyses

GraphPad Prism 8 was used to perform statistical analysis and design graphs. The results are presented as the mean ± SEM of three independent experiments. Statistical analyses between control and treatment conditions were achieved with one-way ANOVA test. Statistical significance was considered when p-value < 0.05, being indicated in graphs as *, **, ***, and **** representing $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

3. Results

3.1. Effects of Edaravone, Perampanel, and Metformin on Cell Viability of HT-22 Cells

3.1.1. HT-22 Cell Viability, Evaluated by MTT Assay, after 6, 24, 48, and 72 h of Treatment

Firstly, we investigated the effects of increasing concentrations, ranging from 0.1 to 100 µM, of edaravone, perampanel, and metformin on HT-22 cells treated for 6, 24, 48, or 72 h. The results are expressed as the percentage of viable cells after 6 (Figure 1A), 24 (Figure 1B), 48 (Figure 1C), or 72 (Figure 1D) hours of treatment.

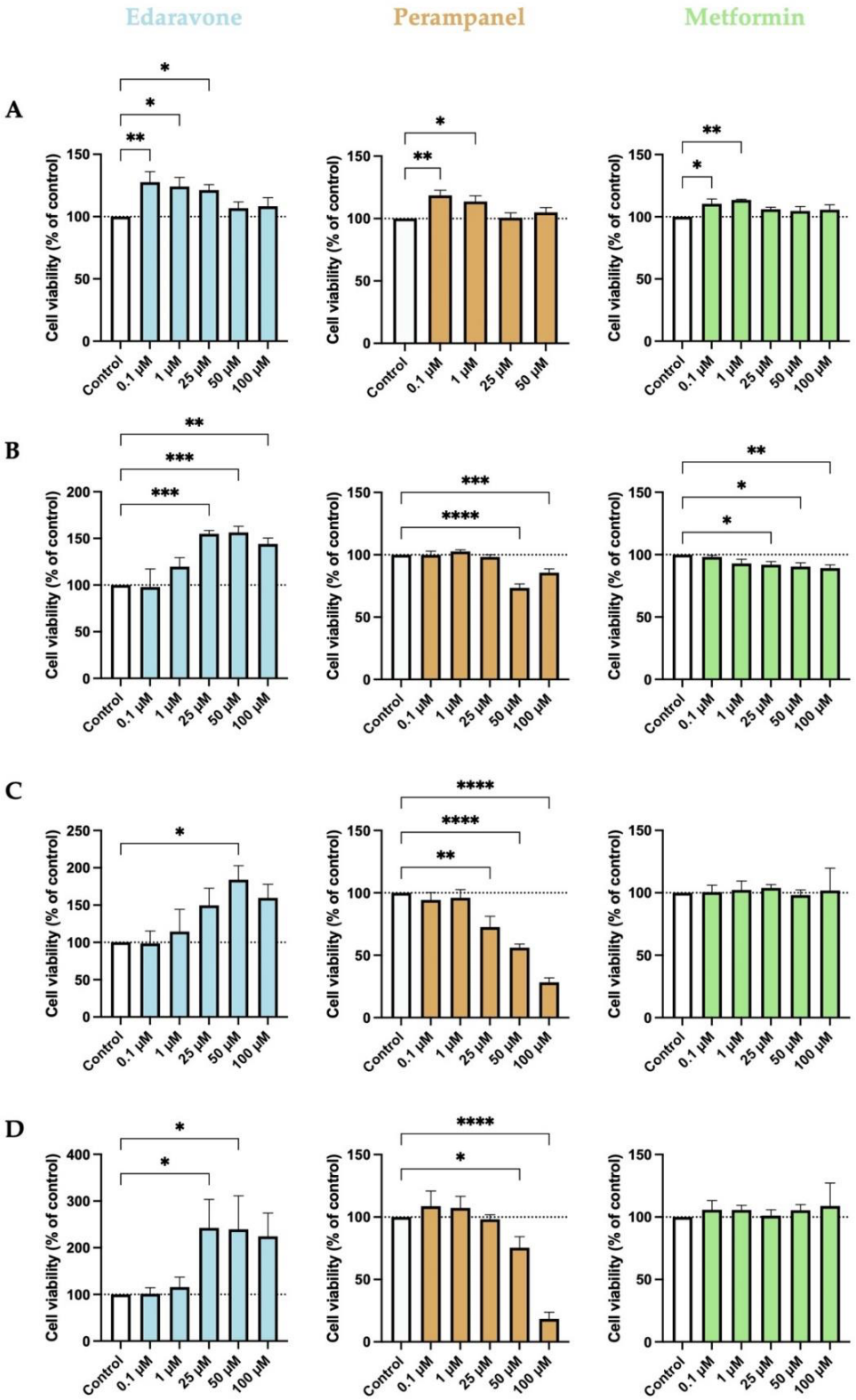


Figure 1. Effects of edaravone, perampanel, and metformin on the viability of HT-22 cells, at different time points of exposure. (**A**) 6-h treatment; (**B**) 24-h treatment; (**C**) 48-h treatment; (**D**) 72-h treatment. Cell viability was assessed by using the MTT assay. Results are expressed as the percentage of cell viability relative to the respective untreated control cells. *, **, ***, and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively, when compared to control. All data are presented as the mean + SEM of three independent experiments.

Our results revealed that the effects of the drugs tested varied as a function of drug concentration and duration of treatment. There was a tendency for edaravone to increase cell viability at all time points when compared to the control. Specifically, after 6 h of treatment, the lower concentrations (0.1, 1, and 25 μM) significantly increased cell viability, an effect that was not apparent when higher concentrations were used. However, after more prolonged treatment periods, particularly after 24 and 48 h, cell viability was significantly increased when the higher concentrations (25, 50, and 100 μM) were used. Relative to perampanel, only the treatment of 6 h with the two lower concentrations significantly increased cell viability. More prolonged treatments had either no effect on cell viability when the lower concentrations were used or significantly decreased cell viability when the higher concentrations were employed. When in lower concentrations (0.1 and 1 μM), metformin significantly increased cell viability only after 6 h of treatment, but it had no effect after more prolonged treatments. A higher concentration of metformin (0.25 to 100 μM) significantly decreased cell viability after 24 h of treatment but had no effect afterward.

The period of 48 h is the most used in this type of experiment since the drug has time to act, and yet there are no major changes in its composition [24]. As expected, 24- and 48-h treatments were the ones that showed better drug activities. Therefore, hereafter, we used 48-h treatment for the main tests.

3.1.2. HT-22 Cell Viability, Evaluated by MTT, SRB and NR Assays, after 48 h of Treatment

We proceed to evaluate whether the assay method influenced the effects of edaravone, perampanel, and metformin on HT-22 cell viability. Thus, we estimated HT-22 cell survival after 48 h of treatment with increasing concentrations of the drugs, using different indirect viability assay methods (MTT, SRB, and NR). The results show that not all assays measure cell viability at equal sensitivity. After 48 h of treatment, edaravone increased cell viability, particularly at high concentrations (50–100 μM), as shown by the MTT and SRB assays, but not by the NR assay (Figure 2A). In contrast, the effect of high concentrations (25–100 μM) of perampanel in decreasing cell viability was apparent using all assays. However, the MTT assay seemed more sensitive in detecting changes between drug concentrations (Figure 2B). Relative to metformin, the results were also similar for MTT, SRB, and NR methods (Figure 2C). However, with this drug, only the NR assay was able to demonstrate a slight, although significant, decrease in cell viability at concentrations higher than 25 μM. Overall, the MTT assay seems to be a more sensitive method to detect small changes for edaravone and perampanel and the NR assay for metformin.

3.1.3. Morphological Analysis of HT-22 Cells after 48 h of Treatment with Increasing Concentrations of Edaravone, Perampanel, and Metformin

As shown in Figure 3, cells submitted to drug treatments display a normal and healthy morphology, except for those treated with higher concentrations of perampanel. After 48 h of treatment with high concentrations of perampanel, HT-22 cells were fewer, compared to the control, indicating a decrease in cell viability. However, treatment with higher concentrations of edaravone (25–100 μM) was associated with a visible increase in cell density. Lastly, treatments with metformin did not appear to induce changes in cell morphology or cell density. Globally, this morphological analysis corroborates the results obtained with MTT, SRB, and NR assay methods, reinforcing that MTT is the most representative viability assay.

3.2. Effects of Edaravone, Perampanel and Metformin on Viability of SH-SY5Y Cells

To complement our results of edaravone, perampanel, and metformin on HT-22 cells, we also tested the same drugs on another cell line, widely used in experimental neurological studies, the SH-SY5Y neuroblastoma cell line. The cytotoxic effects detected after 48 h of treatment using the MTT assay were globally similar to those observed in HT-22 cells (Figure 4). There was a tendency for edaravone to increase cell viability at higher concentrations, but the differences were significant relative to the respective controls only

at concentrations of 100 µM. Similar to HT-22 cells, perampanel also significantly decreased cell viability at higher concentrations (50–100 µM), compared to the respective control. Again, and similar to HT-22 cells, treatment with different concentrations of metformin in this cell line did not result in any significant variation in cell viability.

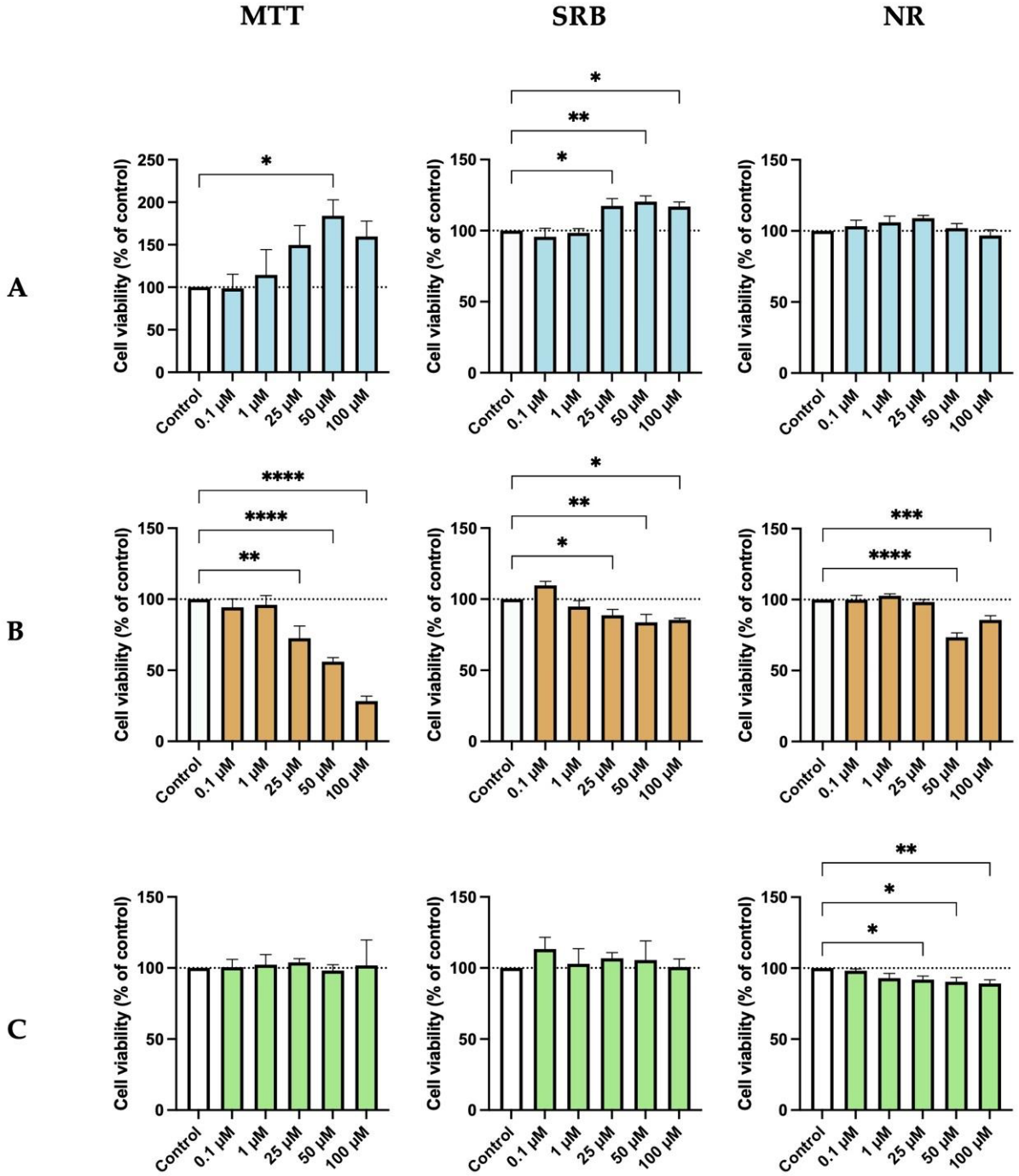


Figure 2. Effects of (**A**) Edaravone, (**B**) Perampanel, and (**C**) Metformin treatment on HT-22 cells viability, evaluated by three different assays. Protein content and cell viability were assessed by SRB (middle panel) and MTT and NR (left and right panel) assays, after 48 h of exposure. Results are expressed as the percentage of cell viability relative to the respective untreated control cells. *, **, ***, and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively, when compared to control. All data are presented as the mean + SEM of three independent experiments.

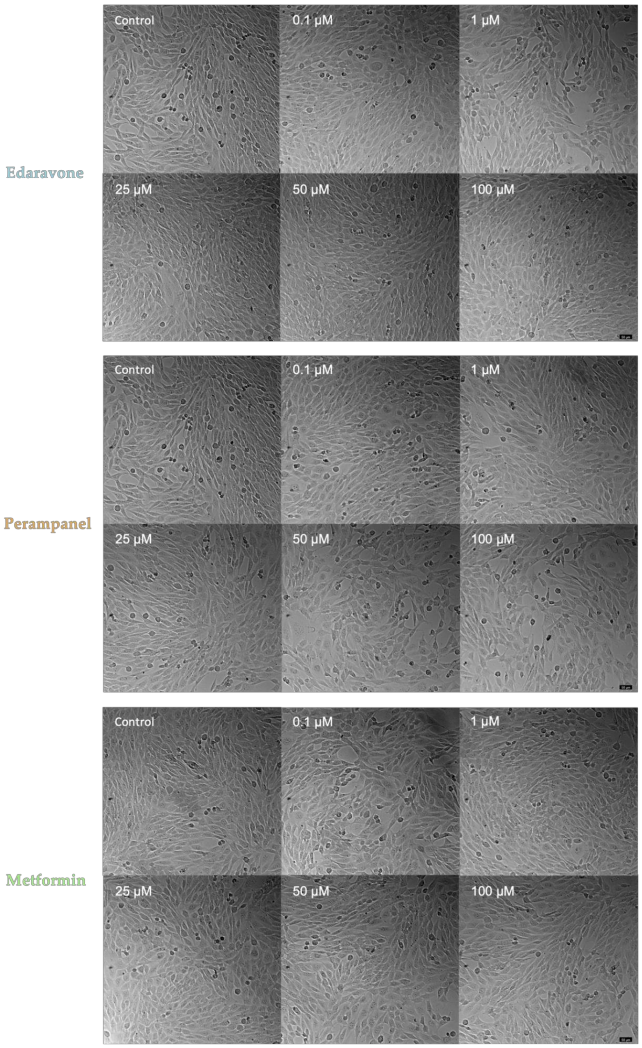


Figure 3. Microscopic representative images of HT-22 control cells and cells after 48-h exposure to concentrations ranging from 0.1 to 100 µM of edaravone, perampanel, and metformin. Images of cell morphology were obtained on a Leica microscope equipped with Leica LAS X imaging software v3.7.4 (Leica Microsystems, Wetzlar, Germany) from three independent experiments. Scale bar: 50 µm.

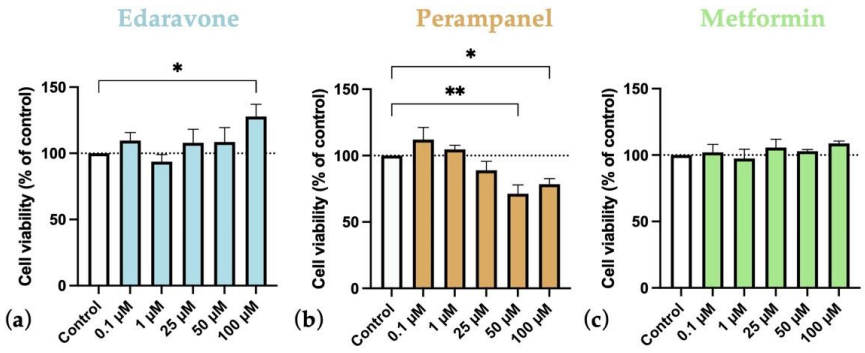


Figure 4. Effects of (**a**) Edaravone, (**b**) Perampanel, and (**c**) Metformin treatment for 48 h on SH-SY5Y cells viability. Cell viability was assessed by MTT assay. Results are expressed as the percentage of cell viability relative to the respective untreated control cells. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, when compared to control. All data are presented as the mean + SEM of three independent experiments.

Morphological Analysis of SH-SY5Y Cells after 48 h of Treatment with Increasing Concentrations of Edaravone, Perampanel, and Metformin

The analysis of the morphology of SH-SY5Y cells (Figure 5) corroborates the cell viability results obtained (Figure 4). Cell density appears to be higher in edaravone-treated cells than controls, but cell morphology is apparently unchanged. Moreover, when in high concentrations, perampanel seems to be cytotoxic because it appears to decrease cell density. Metformin treatments did not significantly alter cell density or morphology. Overall, the morphological analyses are in accordance with data obtained on the viability of SH-SY5Y cells, evaluated using the MTT assay.

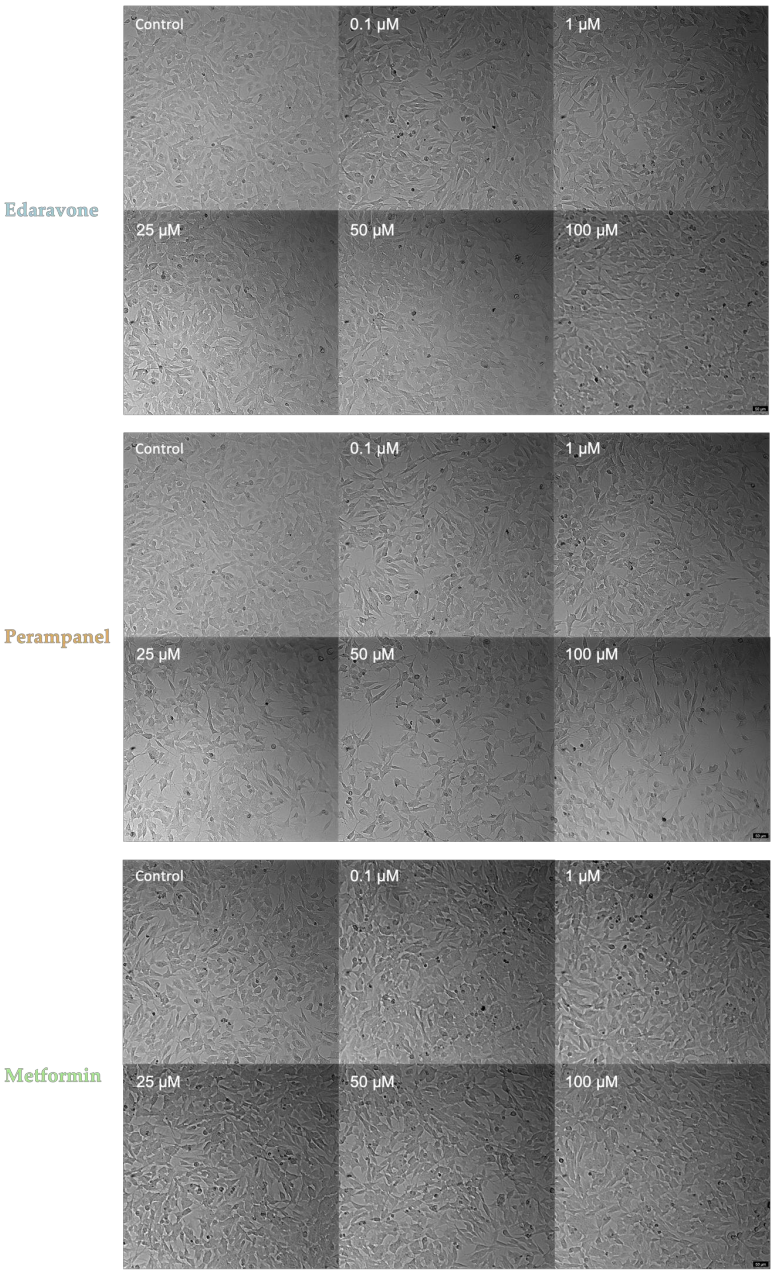


Figure 5. Microscopic representative images of SH-SY5Y control cells and SH-SY5Y cells exposed for 48 h to concentrations, ranging from 0.1 to 100 µM, of edaravone, perampanel, and metformin. Images of cell morphology were obtained using a Leica microscope equipped with Leica LAS X imaging software v3.7.4 (Leica Microsystems, Wetzlar, Germany) from three independent experiments. Scale bar: 50 µm.

3.3. Effects of Hypoxia on HT-22 and SH-SY5Y Cells

We also analyzed treatment effects with increasing concentrations of edaravone, perampanel, and metformin, for 48 h, on both HT-22 and SH-SY5Y cells, maintained for 48 h, under hypoxia conditions. The cell viability results, estimated using the MTT assay, and cell morphology were compared with data obtained from cells of the same lines maintained under a normoxia environment.

3.3.1. HT-22 Cells under Hypoxia Conditions

Our results showed that hypoxia, by itself, reduced HT-22 cell viability (Figures 6 and 7). After 48 h of hypoxia (Figure 6), all controls under hypoxia showed a significant reduction in cell viability compared to the normoxia control (Figure 6a). After 48 h of treatment, on HT-22 cells under hypoxia with edaravone, there was an increase in cell viability that reached significant levels in cells treated with higher concentrations (50–100 μM) (Figure 6b). There was a similar trend for cells treated with perampanel (Figure 6c) and metformin (Figure 6d), particularly at lower concentrations (0.1–1 μM), but the differences did not reach statistically significant levels.

In another experiment delineated to analyze the second phase of hypoxia–ischemia brain injury and correlate with the injury in humans (first, the injury happens, and only after, the treatment is started), we applied the same drugs for 24 h after only 24 h of hypoxia (Figure 7). It was observed that 24-h hypoxia tends to decrease cell viability, albeit only significantly for hypoxia-control but not for the other vehicle controls (Figure 7a). Interestingly, edaravone significantly increased cell viability, even when administered at concentrations ranging from 25 to 100 μM (Figure 7b). Conversely, both perampanel (Figure 7c) and metformin (Figure 7d) treatments had no beneficial effects on cell viability. Metformin even significantly decreased cell viability when applied for 24 h after 24 h of hypoxia.

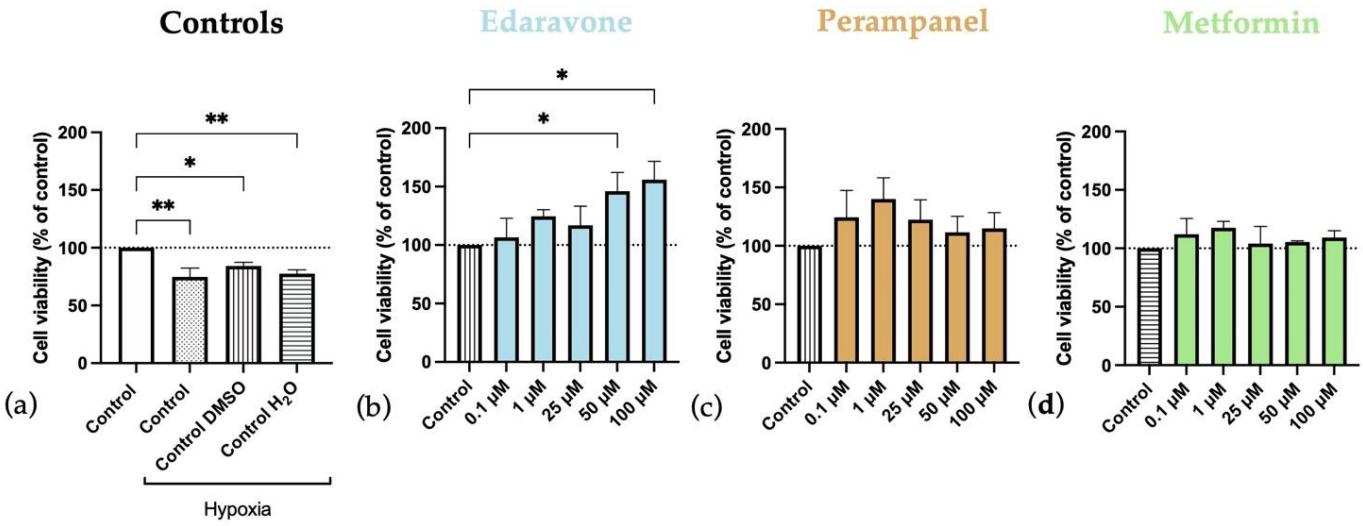


Figure 6. Effects of hypoxia, and edaravone, perampanel, and metformin treatments on HT-22 cells viability. Hypoxia and drugs were applied simultaneously for 48 h. (**a**) Controls graph, comparing controls/vehicles under hypoxic conditions to normoxia ones. (**b**) Edaravone, (**c**) Perampanel, and (**d**) Metformin treatments, while under hypoxia. Cell viability was evaluated by using the MTT assay. Results are expressed as the percentage of cell viability relative to respective untreated hypoxia control cells. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, when compared to the respective control under hypoxia conditions. All data are presented as the mean + SEM of three independent experiments.

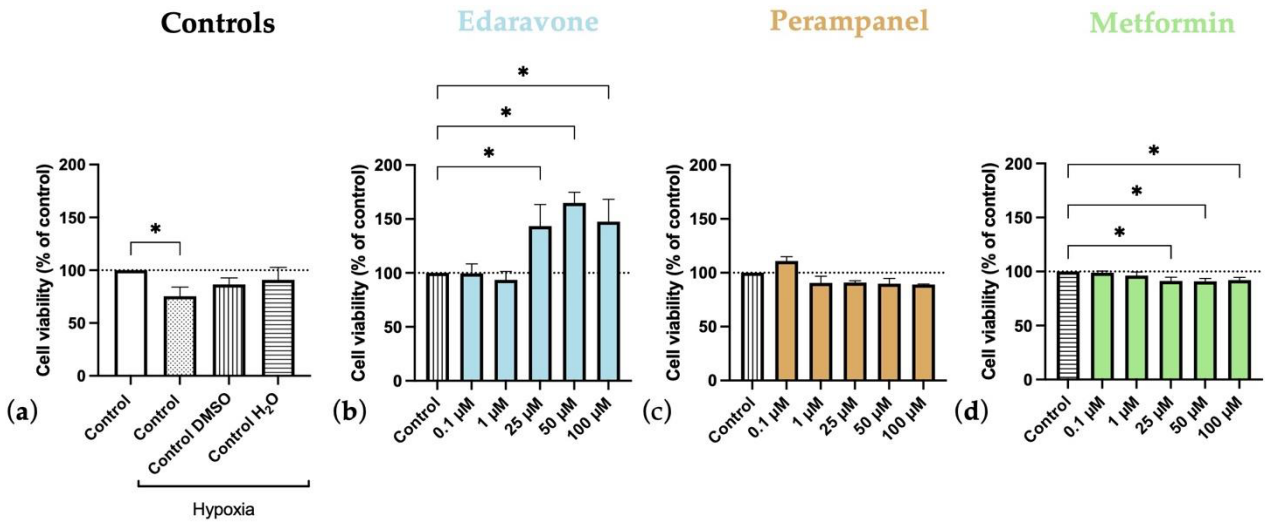


Figure 7. Effects of 24 h of hypoxia followed by 24-h exposure to edaravone, perampanel, and metformin on HT-22 cells viability. (**a**) Controls graph, comparing controls/vehicles in hypoxic conditions to normoxia ones. (**b**) Edaravone, (**c**) Perampanel, and (**d**) Metformin treatments, after 24 h under hypoxia. Cell viability was assessed by MTT assay. Results are expressed as the percentage of cell viability relative to the respective untreated hypoxia control cells in (**b**–**d**). * $p < 0.05$ when compared to hypoxia control. All data are presented as the mean + SEM of three independent experiments.

Moreover, after 48 h of hypoxia, it was clearly visible that there were both morphological and cell density differences between cells in normoxia and hypoxia. Specifically, the cell density was lower in those submitted to hypoxia, and these cells displayed a more rounded shape (Figure 8).

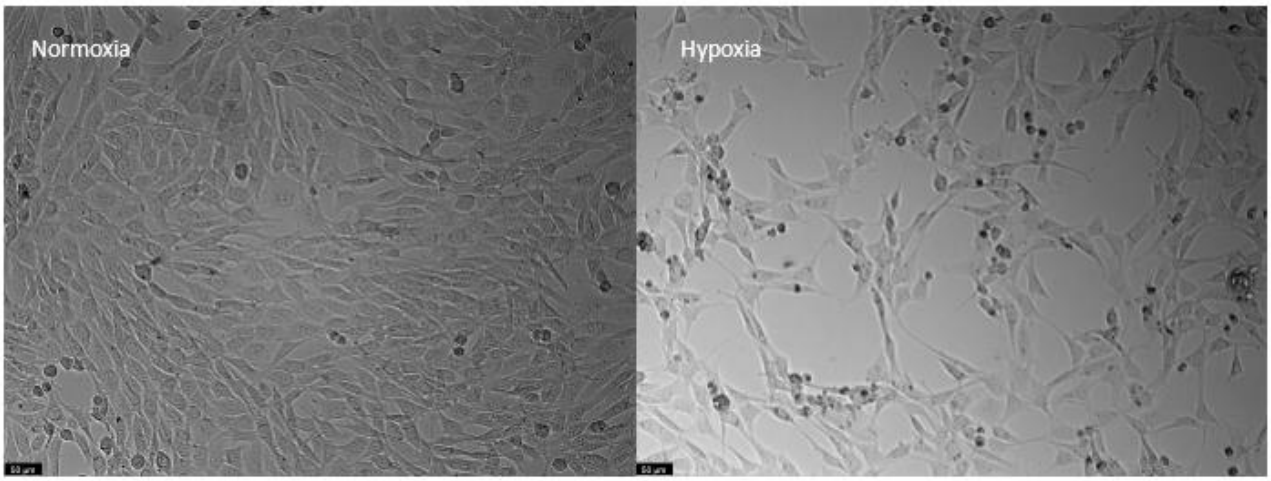


Figure 8. Representative images of HT-22 cells submitted to normoxia (**left**) and hypoxia (**right**) conditions for 48 h. Images of cell morphology were obtained using a Leica microscope equipped with Leica LAS X imaging software v3.7.4 (Leica Microsystems, Wetzlar, Germany). Scale bar: 50 µm.

3.3.2. Morphological Analysis of HT-22 Cells after 48 h of Treatment with Edaravone, Perampanel, and Metformin under Hypoxia Submission for 48 h

As shown globally in Figure 9, treated cells displayed a higher cell density and a more normal cell morphology. Namely, when compared to the respective hypoxia controls, edaravone and perampanel higher (50–100 µM) and lower concentrations (0.1–1 µM), respectively, promoted cell density increase and a healthier morphology appearance. These morphological results corroborate cell viability results described in Figure 6. On the other

hand, the hypoxia cells treated with metformin appeared to increase cell density, which is not as visible and accentuated in the cell viability plot in Figure 6.

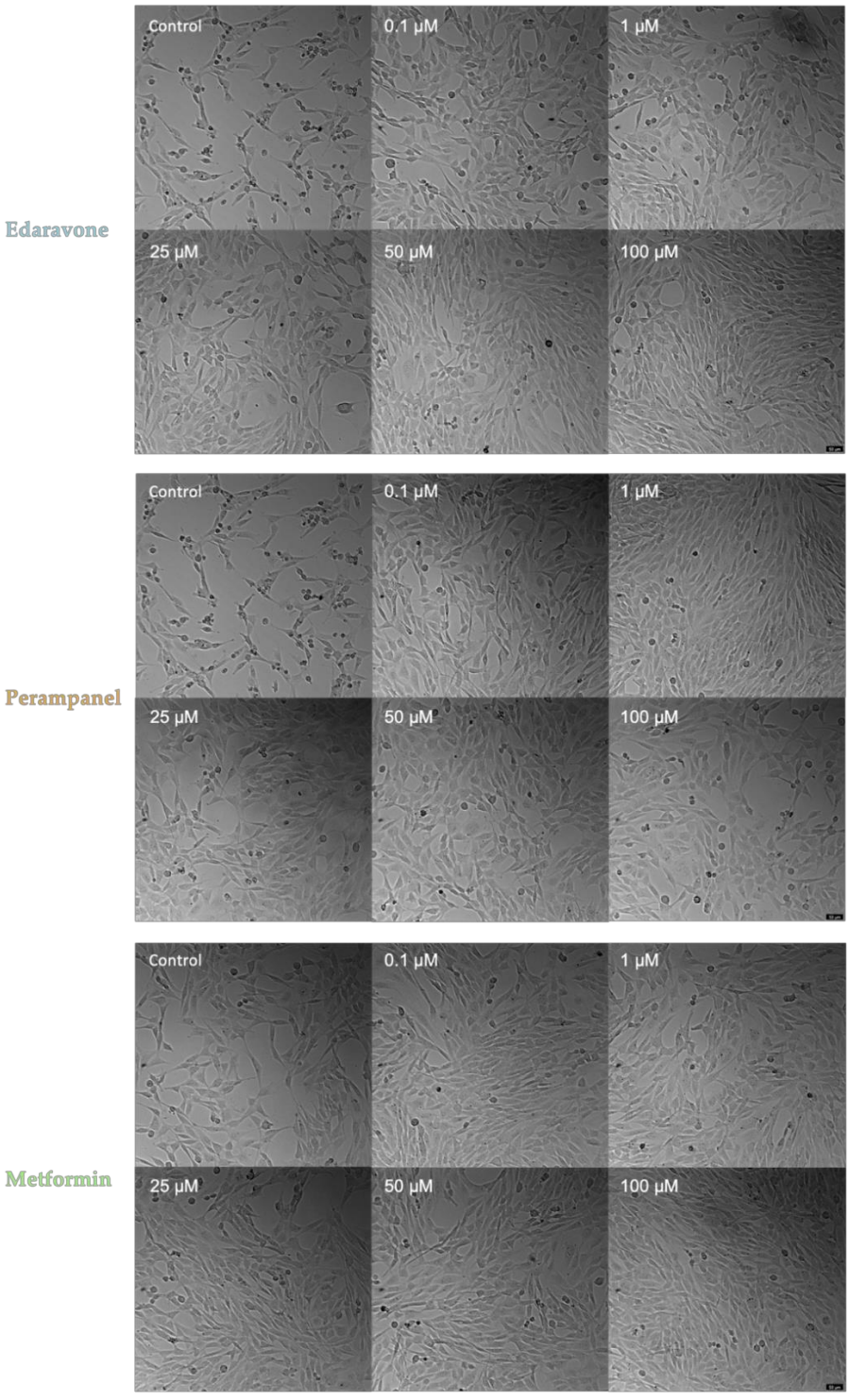


Figure 9. Microscopic representative images of HT-22 hypoxia control cells and HT-22 cells exposed for 48 h to hypoxia and drug concentrations, ranging from 0.1 to 100 µM, of edaravone, perampanel, and metformin. Images of cell morphology were obtained using a Leica microscope equipped with Leica LAS X imaging software v3.7.4 (Leica Microsystems, Wetzlar, Germany) from three independent experiments. Scale bar: 50 µm.

3.3.3. SH-SY5Y under Hypoxia Conditions

The response to hypoxia of SH-SY5Y cells was different from that observed for HT-22 cells. Specifically, hypoxia did not reduce cell viability, and it even showed a trend towards increasing it, albeit not significantly, as can be seen in the control cell viability graph (Figure 10a) and in the respective representative images (Figure 11). Consequently, drug treatment (Figure 10) had no major effects on cell viability, except for 50 µM of perampanel, which significantly reduced cell viability. Regarding cell morphology, in hypoxia conditions, they appear to be as expected, pyramidal; however, it is visible that some are more rounded. These results suggest that SH-SY5Y can not only survive but even thrive in a hypoxic environment.

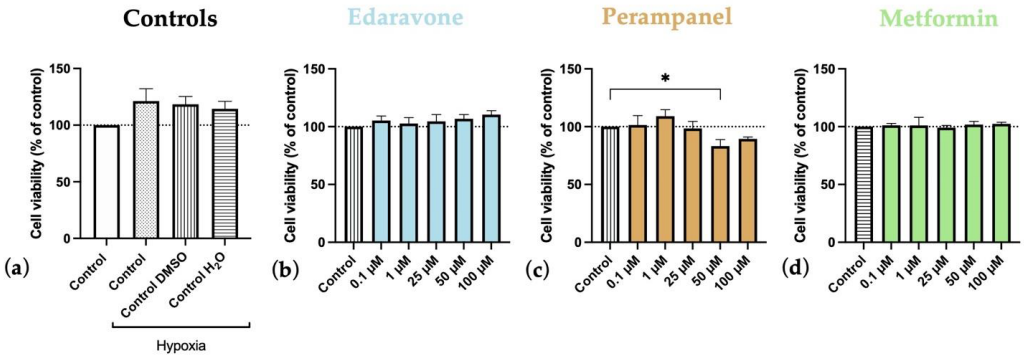


Figure 10. Effects of hypoxia, and edaravone, perampanel, and metformin treatments on SH-SY5Y cells viability. Hypoxia and drugs were applied simultaneously for 48 h. (**a**) Controls graph, comparing controls/vehicles in hypoxic conditions to normoxia ones. (**b**) Edaravone, (**c**) Perampanel, and (**d**) Metformin treatments, while in hypoxia. Cell viability was assessed by MTT assay, to each treatment after exposure. Results are expressed in the percentage of cell viability relative to untreated hypoxia-control cells and submitted to hypoxia in (**b**–**d**). * indicate $p < 0.05$, when compared to hypoxia-control. All data are presented as the mean + SEM of three independent experiments.

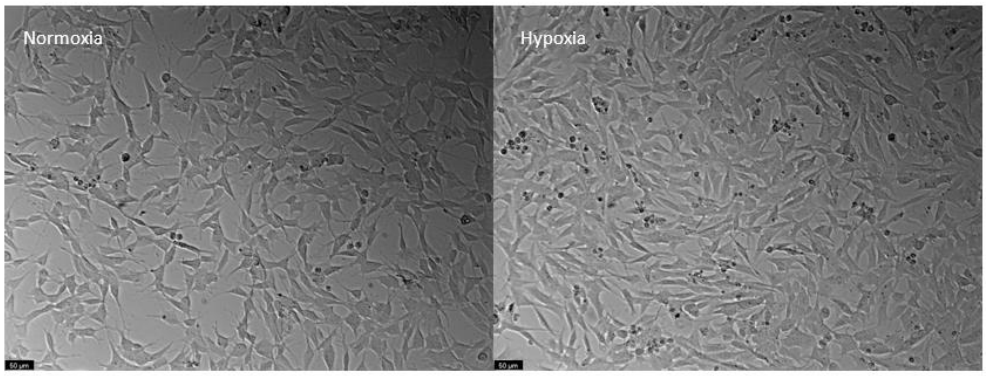


Figure 11. Representative images of SH-SY5Y cells submitted to normoxia (**left**) and hypoxia (**right**) conditions for 48 h. Images of cell morphology were obtained using Leica microscope equipped with Leica LAS X imaging software v3.7.4 (Leica Microsystems, Wetzlar, Germany). Scale bar: 50 µm.

3.4. Effects of Oxygen-Glucose Deprivation on HT-22 Cells

OGD represents a severe challenge for cells, mimicking hypoxia–ischemia insults. Our results show that OGD significantly decreased the viability of HT-22 cells in all control groups, both after 6 (Figure 12A) and 48 (Figure 12B) hours of deprivation. As expected, the decrease was more severe after 48 h than after 6 h of deprivation.

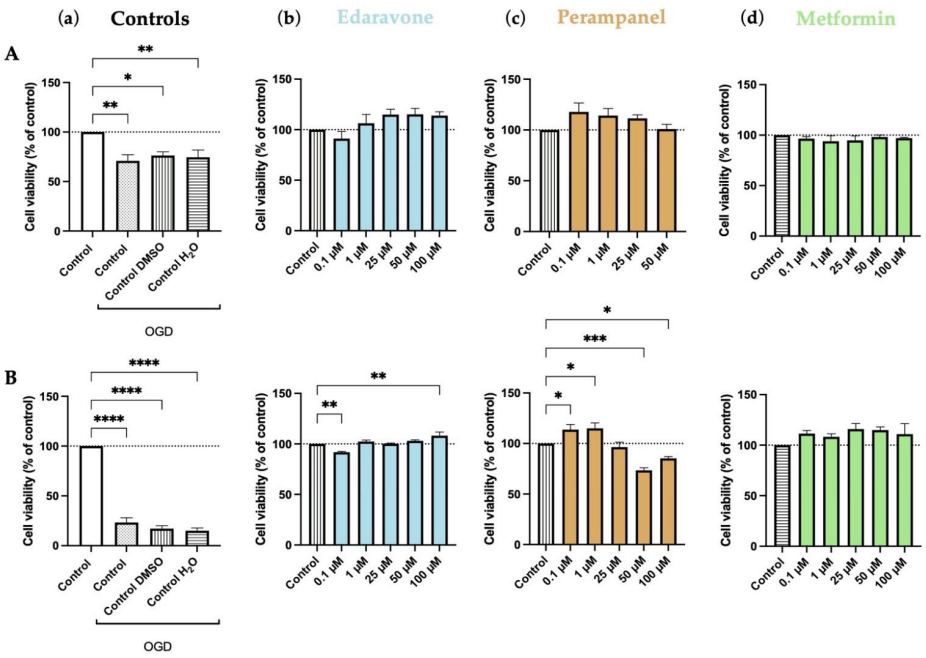


Figure 12. Effects of oxygen–glucose deprivation (OGD) and edaravone, perampanel, and metformin treatments for 6 (**A**) and 48 (**B**) hours on HT-22 cells viability. (**a**) Controls graph, comparing controls/vehicles in OGD conditions to standard medium. (**b**) Edaravone, (**c**) Perampanel, and (**d**) Metformin treatments, while in OGD conditions. Cell viability was assessed by MTT assay. Results are expressed as the percentage of cell viability relative to untreated control cells submitted to OGD. *, **, *** and **** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, when compared to the respective control. All data are presented as the mean + SEM of three independent experiments.

During the 6-h period, edaravone and perampanel showed a trend to increase cell viability, compared to the respective OGD-treated control. However, only in the 48-h period did the differences achieve significant levels in some concentrations (100 µM for edaravone and 0.1–1 µM for perampanel; Figure 12). As previously shown, perampanel at higher concentrations reduced cell viability. Metformin did not significantly alter the viability of HT-22 cells under OGD conditions (Figure 12).

The morphology of cells submitted to OGD was altered. Cells showed a smaller and more rounded shape, with longer prolongations than controls. Their density was also clearly reduced (Figure 13).

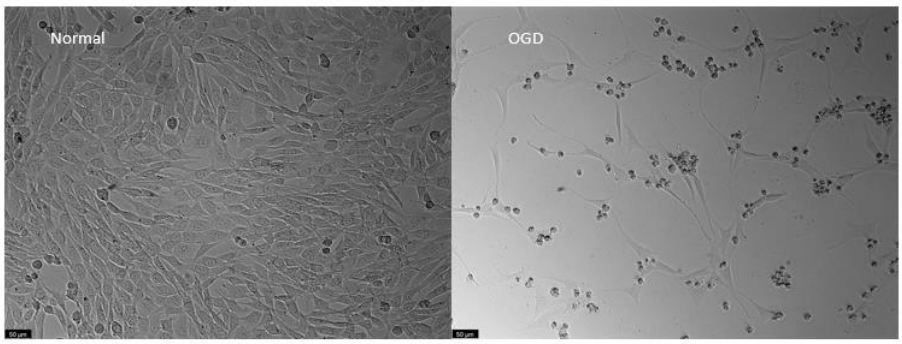


Figure 13. Representative images of HT-22 cells morphology, both under normal or Oxygen-Glucose Deprivation conditions during 48 h. Images of cell morphology were obtained using a Leica microscope equipped with Leica LAS X imaging software v3.7.4 (Leica Microsystems, Wetzlar, Germany). Scale bar: 50 µm.

Morphological Analysis of HT-22 Cells after 6 and 48 h of Treatment with Edaravone, Perampanel, and Metformin under Oxygen Glucose Deprivation Submission

Figures 14 and 15 show treated HT-22 cells morphology that were subjected to OGD for 6 and 48 h, respectively. The cells subjected to 6 h of OGD appear to undergo morphological changes, although these are not as prominent as those observed from the extensive injury caused by 48 h of OGD. In the 6-h treatment images, slight changes in perampanel cell density were observed, with an explicit increase of 0.1 µM, which is in accordance with the graphs in Figure 12A.

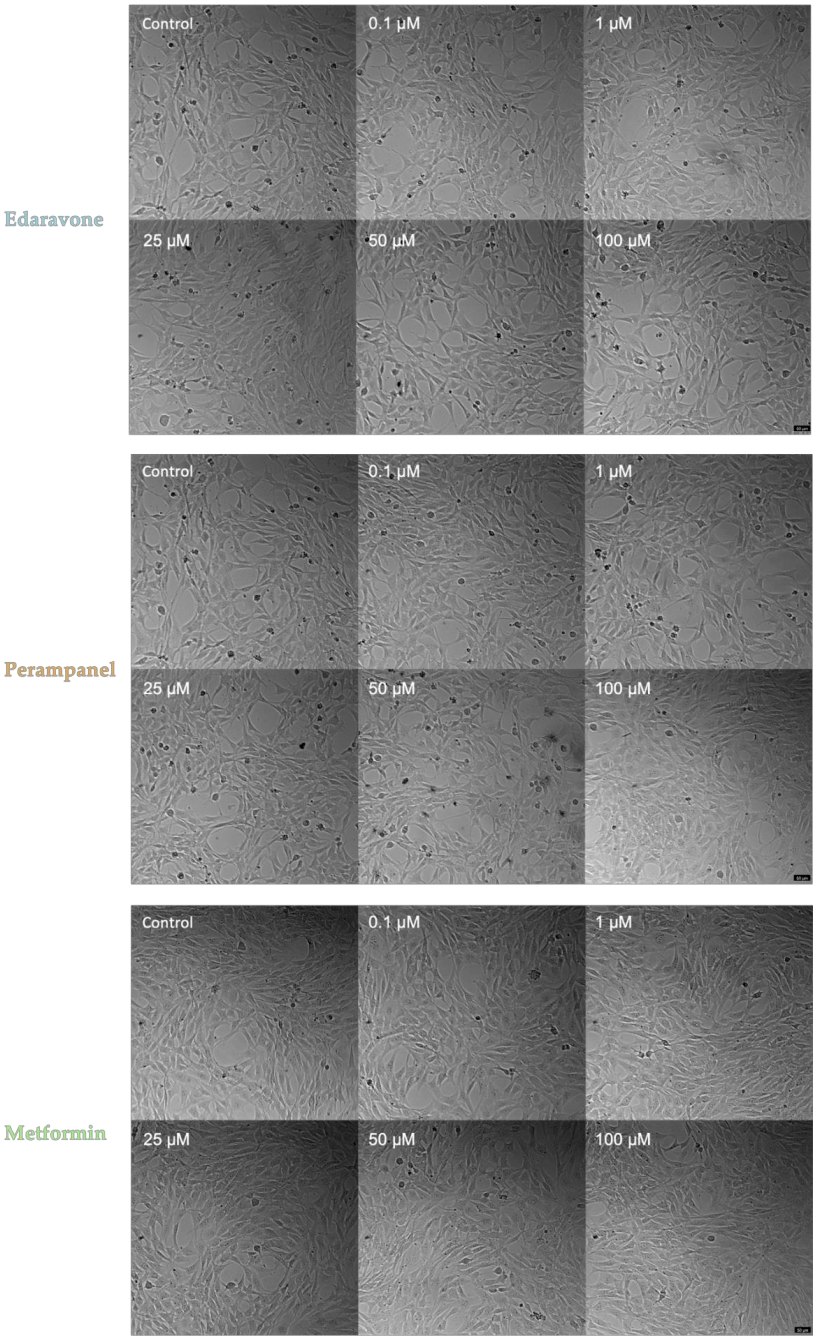


Figure 14. Microscopic representative images of HT-22 oxygen–glucose deprivation (OGD) control cells and HT-22 cells exposed for 6 h to OGD and concentrations, ranging from 0.1 to 100 µM, of edaravone, perampanel, and metformin. Images of cell morphology were obtained using a Leica microscope equipped with Leica LAS X imaging software v3.7.4 (Leica Microsystems, Wetzlar, Germany) from three independent experiments. Scale bar: 50 µm.

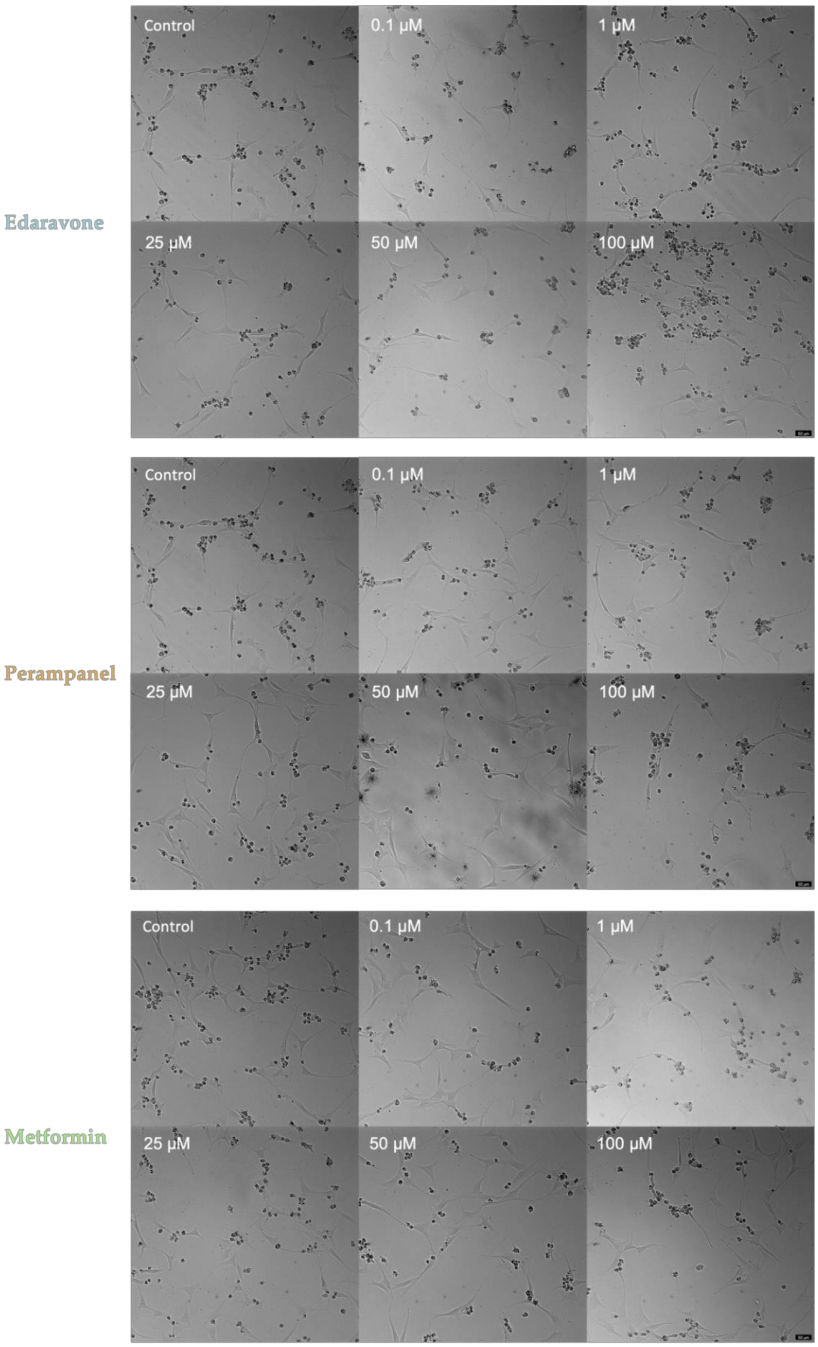


Figure 15. Microscopic representative images of HT-22 oxygen–glucose deprivation (OGD) control cells and HT-22 cells exposed for 48 h to OGD and concentrations, ranging from 0.1 to 100 µM, of edaravone, perampanel, and metformin. Images of cell morphology were obtained using a Leica microscope equipped with Leica LAS X imaging software v3.7.4 (Leica Microsystems, Wetzlar, Germany) from three independent experiments. Scale bar: 50 µm.

Despite the results from Figure 12B, there were no major changes, especially in morphology (Figure 15), between the different drug concentrations and the respective controls. However, the representative image of 100 µM edaravone treatment showed a greater number of rounded cells, which could be the reason for the statistical significance in Figure 12B. Finally, regarding morphological analysis, the individual administered drugs could not recover the cells to a healthy and normal morphology when submitted to an OGD insult.

3.5. Reactive Oxygen Species Measured on HT-22 Cells Submitted to OGD and Treated with Edaravone, Perampanel, and Metformin

Reactive oxygen species can be estimated and detected using DCFH-DA dye, which becomes fluorescent with ROS generation. This experiment was performed with time points of 6, 24, and 48 h.

As expected, ROS production was increased, at all different time points, in OGD conditions. Notably, treatment of HT-22 cells submitted to OGD with the three drugs decreased ROS production, when compared to the control group. However, the decrease was greater with edaravone, where we observed significantly lower levels of ROS when cells were treated with higher concentrations of edaravone. This result reinforces the view that edaravone may protect cells submitted to hypoxic insults. Perampanel and metformin decreased ROS production when compared to the control. However, as the concentration increases for both of them, there is also an increase in ROS production, indicating that higher concentrations may induce cytotoxic effects. (Figure 16).

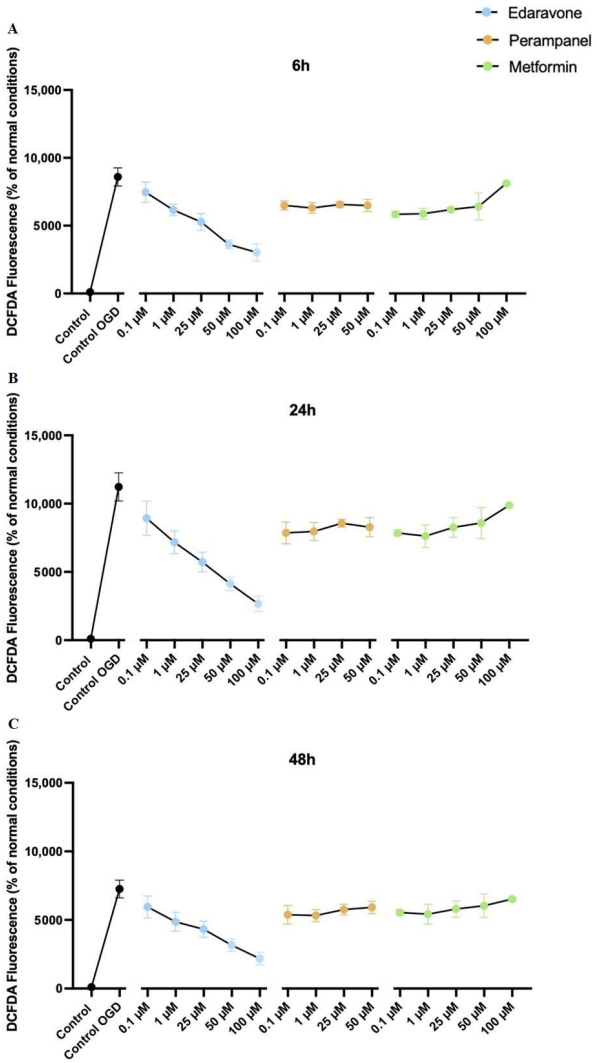


Figure 16. Protective efficacy of edaravone, perampanel, and metformin in HT-22 cells exposed to oxygen–glucose deprivation (OGD) over 6 (**A**), 24 (**B**), and 48 (**C**) hours. DCFDA fluorescence (dye of ROS generation) change percentage (%) of the different concentrations of edaravone, perampanel, and metformin, exposed to OGD. Results are expressed as the percentage of DCFDA fluorescence relative to control cells, in normal conditions. Results were assessed by fluorescence plate reader (SpectraMax Gemini EM Microplate Reader, Molecular Devices, San Jose, California), 485 nm excitation and 530 nm emission. Data are presented as the mean ± SEM of three independent experiments.

Since edaravone was the drug that stood out as the most efficient in decreasing ROS production, we evaluated its effects in a more detailed way. As observed in Figure 17a, 6 h of treatment was not enough to significantly decrease ROS production in cells submitted to OGD. However, after 24 h (Figure 17b) as well as after 48 h (Figure 17c) of treatment, all edaravone concentrations were able to significantly decrease ROS detection in HT-22 cells submitted to OGD insult. These results reinforce the view that edaravone may protect cells submitted to hypoxic insults, possibly by reducing toxic species production in hypoxia injured HT-22 cells.

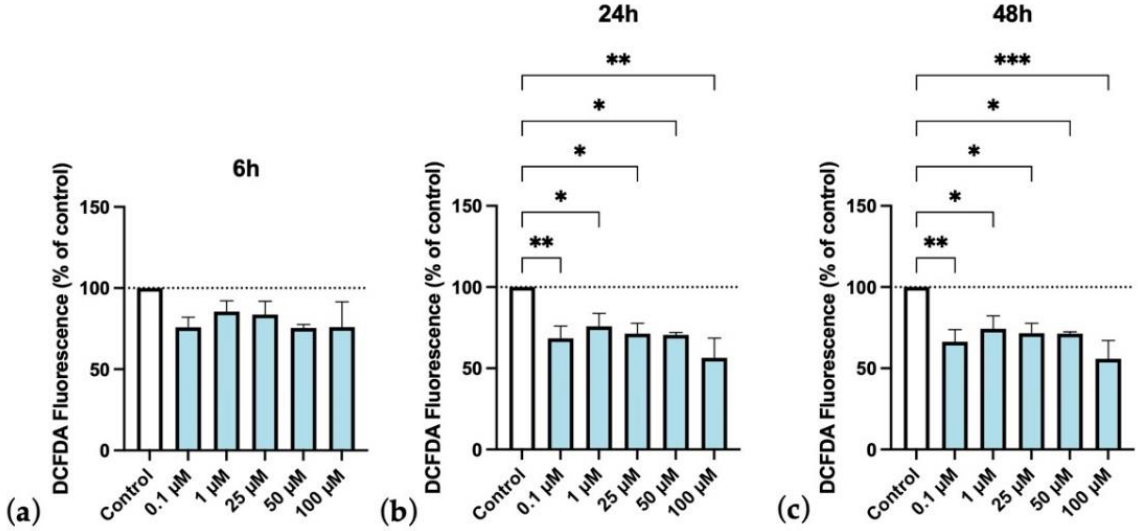


Figure 17. Protective efficacy of edaravone on HT-22 cells exposed to oxygen–glucose deprivation (OGD), over time (6, 24, and 48 h, (**a**), (**b**), and (**c**), respectively). DCFDA fluorescence (dye of ROS generation) change percentage (%) of the different concentrations of edaravone exposed to OGD conditions over control. Results are expressed as the percentage of DCFDA fluorescence relative to untreated control cells in OGD. Results were assessed by fluorescence plate reader (SpectraMax Gemini EM Microplate Reader, Molecular Devices, San Jose, California), 485 nm excitation and 530 nm emission. All data are presented as the mean + SEM. *, **, and *** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, when compared to control.

4. Discussion

Neonatal brain injury due to hypoxia–ischemia is an important health problem and is associated with severe neurologic disabilities. Despite advances in the knowledge about the effects of hypoxia–ischemia events in the brain, clinically approved therapeutic interventions for preterm and neonatal hypoxia–ischemia are scarce. In this way, we aimed to test three repurposed drugs commonly used for brain disease treatments, to see if they could revert or at least attenuate hypoxia–ischemia-induced alterations. We used edaravone, perampanel, and metformin, which have already been approved for the treatment of several pathologies and seem to have interesting properties in oxygen and glucose deprivation, namely hypoxia–ischemia insults.

Firstly, we tested how the hippocampal HT-22 cells and SH-SY5Y cells react to the treatment with edaravone, perampanel, and metformin. Among the panoply of brain cell lines, we chose the HT-22 cell lines because it is an established cell line to study neurotoxicity and also because we aim to study, in the future, the potential beneficial effects of these drugs on the treatment of the well-known impairment of learning and memory processes associated with hypoxia–ischemia events [25], particularly in prematurity [26]. Moreover, and for comparative purposes, we decided to test the same drugs in another brain cell line, namely the neuroblastoma SH-SY5Y cell line, to examine whether the potential effect of these drugs is global or, otherwise, specific to some neuronal cell lines. In this way, we tested if these drugs, in a vast range of concentrations and duration of exposure, were cytotoxic to the HT-22 and SH-SY5Y cell lines.

Not surprisingly, we found that edaravone increased cell viability in both types of cells at all time points compared to the control. Our data corroborate several previous reports that have consistently demonstrated that edaravone increases cell viability, has neuroprotective properties [27–29], and is a safe drug, at least for the types of cell lines herein studied. Interestingly, both perampanel and metformin had the capacity to increase cell viability when applied during a short period (6 h) and at low concentrations (0.1–1 μM). However, they had no impact on cell viability at medium concentrations and were cytotoxic at higher concentrations (more than 50 μM) and more prolonged treatments (more than 24 h), particularly for perampanel. Although not as efficient as edaravone, metformin and perampanel also can increase cell viability, which is in line with data from previous studies [30,31]. Although these effects of perampanel were particularly obvious when using the MTT assay, which we have demonstrated to be more precise and sensitive to small changes in both cell lines, they were similar to those obtained with the other viability assays used in the present study.

After we tested the cytotoxicity of the present drugs, we then proceeded to evaluate their potential effects in hypoxia–ischemia conditions. In the present work, we used two cellular models of hypoxia–ischemia, one with a hypoxic atmosphere and another that included the association between a hypoxic atmosphere and glucose deprivation. We found that both hypoxic atmosphere and oxygen–glucose deprivation significantly reduced HT-22 cell viability and induced noticeable alterations in their morphology, which was dependent on the time of exposure, corroborating previous studies that have also used hypoxia [32] and oxygen–glucose deprivation [33] cell models. Interestingly, in the neuroblastoma cell line, the hypoxia conditions increased cell viability, despite inducing small morphological changes. Although, we were expecting that this degree of hypoxia could induce significant alterations, even in neuroblastoma cells. This finding was not completely surprising, since it was previously found that hypoxia could favor neuroblastoma cell line survival and proliferation [34–36]. Indeed, this could be explained by the activation of different transcriptional programs driven by hypoxia-inducible factors (HIFs) [37] affecting the cell cycle and proliferative status. The HIFs' effects are context and cell type dependent. In particular, in tumor cells, the genes regulated by these molecules are highly expressed, allowing adaptation mechanisms [38]. Nevertheless, despite the absence of significant alterations in neuroblastoma cells using the present hypoxia model, we decided to test the efficiency of edaravone, metformin, and perampanel also in these cells to see if these drugs were able to improve cell viability during and after the insults.

Among the drugs used in the present study, edaravone was the most efficient in treating the hypoxia-related effects. Indeed, after using this drug, we observed a significant attenuation of hypoxia and oxygen–glucose deprivation effects, demonstrated by a significant increase in cell viability and by a decrease in ROS production, corroborating previous studies showing the efficacy of this drug in hypoxia in vitro [28,39] as well as in vivo models [40–42]. We believe that these results support the effects of edaravone on hippocampal HT-22 cells under hypoxic conditions, showing that this drug is very efficient in attenuating the impact of hypoxia in hippocampal cells. Our data also lend support to previous studies showing that edaravone attenuates hypoxia-induced hippocampal damage and cognitive impairment [40]. One mechanism that could explain edaravone's protective effects on HT-22 cells, in hypoxic conditions, is its free radical scavenging activity [10]. This not only suggests that edaravone activates neuroprotective mechanisms against OGD insults, but also highlights the role of ROS in brain injuries associated with hypoxia–ischemia. Indeed, previous studies have already demonstrated that edaravone attenuates hypoxia-induced neuronal damage via ROS scavenging and upregulation of CREB phosphorylation [40], as well as by reducing apoptotic events and inhibiting hypoxia-inducible factor-1α and cleaved caspase-3 protein expression [41]. In this way, it is tempting to speculate that the alterations of the neurogenic process that we have found in a hypoxia–ischemia in vivo model of previous work, of our group [43], it may be, also, ascribed to changes in these pathways.

Relative to perampanel, an antagonist of AMPA receptors, we have found that this drug, when in low concentrations, exerts beneficial effects against both hypoxia and OGD insults. Whereas in higher concentrations, it decreases cell viability and increases ROS production. Thus, it seems that when in low doses, perampanel may protect neuronal cells from hypoxia and OGD, potentially due to its capability of reducing neuronal overexcitation [13], necroptosis, and neuroinflammatory events [30]. However, it seems that to be efficient, perampanel needs to be administered in low concentrations and simultaneously with hypoxic events. Interestingly, in the present experiment, perampanel was the only drug that increased the viability of neuroblastoma SH-SY5Y cells in normal as well as under hypoxia conditions. However, when in high concentrations, perampanel was not efficient in our hypoxia models, probably because higher concentrations alter normal cell functions and basal excitatory transmission, as previously described [44].

Finally, data obtained in this study indicate that metformin is ineffective in attenuating hypoxic events. This work focused on the insults and treatments in the first phase of the hypoxia–ischemia evolving process. We did not address the reperfusion phase when oxygen and glucose are restored, and inflammatory events are thought to be the major cause of injury. It may be due to this reason that metformin did not show noteworthy results since it is recognized as an anti-inflammatory drug. Indeed, this assumption is partially supported by our finding that metformin was more effective after 48 h of oxygen glucose deprivation than after 6 h of treatment.

By establishing a hypoxia–ischemia model in neuron-like cells, the doors are open to study drugs or compounds that may be able to attenuate the resulting damage and give light on future therapeutic options for hypoxia–ischemia brain injuries. In summary, this work allowed us to demonstrate that edaravone, perampanel, and metformin repurposed drugs are of interest in the early stages of hypoxia–ischemia brain injuries, such as encephalopathy of prematurity. In addition, it also confirmed that ROS production and overexcitation play an important role in the development of the injury. More studies must be performed, mostly in vivo, to confirm our results in a more translational analysis. Furthermore, these three drugs need to be further studied to better understand their mechanism of action and test if their mechanisms of action do not interfere with normal cell function.

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DISCUSSION

The main goal of the present thesis was to better understand the long-term neurologic impact of early hypoxia-ischemia in children born preterm and at term.

We verified, in different clinical scenarios (PVL, perinatal stroke, HIE, encephalopathy of prematurity) that hypoxia-ischemia injury, in the neonatal period, is responsible for adverse neurologic outcomes later in life. The severity and nature of the outcome are dependent on the gestational age at the time of the insult and the type and duration of the insult. From preterm to term age, ischemia results in motor and non-motor impairments that remain in time and limit the quality of life.

In the following paragraphs, we will describe the odyssey we took to characterize the paediatric brain subjected to a neonatal ischemic insult. For a global overview, we decided to capture the clinical perspective, dive into the experimental neuropathological mechanisms and emerge with new treatments.

OUTCOME OF CHILDREN WITH PERIVENTRICULAR LEUKOMALACIA AND THE RELATION WITH PRENATAL EXPOSURE TO ISCHEMIA AND INFECTION

To start this odyssey and get a general overview we conducted a retrospective review of medical records of children with PVL and analysed the role of infection and ischemia in the neurodevelopmental outcomes of these children. We have found that only about one-quarter of the children had a normal developmental outcome, with the remainder having different grades of developmental impairment. Furthermore, children with moderate to severe PVL were significantly more impaired than children with mild PVL. Indeed, we have found that about 70% of the cases developed cerebral palsy, 29% of children had epilepsy and 15% were microcephalic, corroborating previous studies where similar results were reported[80–82]. Intending to clarify the weight of ischemia and infection in the prognosis of PVL, we classified the children according to the predominance of ischemic or infection risk factors. Interestingly, we found that the percentage of moderate to severe PVL was superior in children with predominant infection risk factors (93.3% vs 71.4%) when compared with children with

ischemic risk factors. Indeed, children in the infectious group were more prone to abnormal development and cerebral palsy, while children in the ischemic group had more epilepsy and hearing impairment, suggesting, in this later group, a more prominent involvement of grey matter. In this way, our results also suggest that, besides the brain region affected, the etiology of the lesion is important in determining the type and severity of deficits. An ischemic lesion may determine a more extensive involvement of grey matter injury, which may lead to other deficits not so common in PVL, like epilepsy.

Regarding placental pathology reports, ischemic lesions were described in only one case versus 11 cases with descriptions suggestive of infection, reinforcing the assumption that infection-induced inflammation early in life (in the uterus) is probably an important contributor to white matter injury seen in preterm children.

Regardless of some limitations of our study, as far as we know, this was the first study trying to identify a relationship between the type of insult (ischemic vs. infectious) and the neurological outcomes of preterm children with PVL. Infection-related inflammation seems to be a strong trigger for PVL, and, therefore, all efforts should be made to prevent infection in preterms. This measure should be considered an additional neuroprotection procedure to be aware of in the neonatal intensive care units.

OUTCOME OF PERINATAL ARTERIAL ISCHEMIC STROKE WITH AN ATTEMPT TO CORRELATE IT WITH IDENTIFIED RISK FACTORS

After investigating ischemia in the preterm, we decided to focus on ischemia in children born at term. Continuing our retrospective studies, we analysed the risk factors, clinical manifestations and follow-up of neonates with perinatal stroke. In our study, we observed that out of 31826 live births, 17 cases of perinatal stroke were identified, corresponding to an estimated incidence of 1 in 1872 live births, and that three-quarters of these cases were ischemic strokes. Thus, this incidence is near the higher values of incidence reported in literature[65,67].

Delivery in perinatal stroke is commonly uneventful and children usually have good Apgar scores, which contrasts with what happens with HIE (difficult delivery and low Apgar scores),

constituting an important clue to differential diagnosis between the two conditions. Not surprisingly, we have found that 88% of the neonates with perinatal stroke developed seizures 20 to 48 hours after delivery.

In an attempt to correlate the outcome of perinatal stroke with identified risk factors, we found that all ischemic stroke cases had at least one risk factor and all cases with more than two risk factors had neurologic sequelae. The combination of risk factors may represent a more severe ischemic insult and, consequently, a more severe injury and worse outcome. Considering the outcome, hemiplegic cerebral palsy was present in 41% of ischemic stroke patients, an abnormal neurodevelopmental assessment was found in 64% and there was no stroke recurrence or death with a mean follow-up of 43 months. These results corroborate data from previous studies (25-50% exhibited hemiplegic cerebral palsy and about 60% experienced cognitive and language impairment)[83]. The risk for post-stroke epilepsy after perinatal stroke is dependent on many variables (e.g. vascular subtype), thus a wide range of prevalence is reported in the literature (15% to 54%)[83,84]. In our cohort, epilepsy was present in 25% of patients. Our study reinforces the importance of closer and prolonged follow-up after perinatal stroke.

CLINICAL OUTCOME OF CHILDREN WHO SUFFERED NEONATAL HYPOXIA-ISCHEMIA ENCEPHALOPATHY BEYOND CEREBRAL PALSY

We continued our journey by investigating the impact of ischemia in term neonates. In the TH era, we evaluated neonatal HIE, in which the outcome was not cerebral palsy. Studying a prospective cohort of children with neonatal HIE at school age, we analysed data from neurological examination and motor function, and the possible correlations with general cognitive abilities and attention at school age, as well as neuromotor assessments and motor development at toddler age and neonatal MRI. Interestingly, we found that HIE children treated with TH, even in the absence of cerebral palsy, can present long-term motor difficulties at school age. In particular, manual dexterity and balance skills difficulties were more frequent in children with HIE than in the comparison group. Remarkably, we have also found that 22% of children with neonatal HIE in the absence of cerebral palsy had MND and showed that MND

was associated with poorer motor skills. The prevalence of motor impairment that we have found is in the range (5-38%) of the prevalence reported in literature[25,85]. Importantly, these results were consistent with parental concerns about motor skills in daily life. Moreover, it appears that inattention may contribute to the impairment of motor performance. Indeed, children with HIE scored significantly higher on the DuPaul ADHD Rating Scale Inattention subscale and children with HIE with higher scores of inattention were more likely to have poorer motor performance. Attention and the ability to plan and execute correctly motor tasks are two functions of the prefrontal cortex, whose volume loss and dopamine disturbance have been proposed as a possible explanation for attention deficits in ischemia in rats[86,87]. Thus, prefrontal cortex dysfunction is probably the neural substrate to explain these deficits in HIE.

In addition, we found that inattention was related to MND, indicating that children with MND are particularly prone to be affected in their performance at school. In this perspective, MND may be seen by physicians as a sentinel sign that can be identified in medical appointments, allowing the selection of children with a high risk of learning difficulties for adequate educational support. Moreover, we also found that in the absence of cerebral palsy, neonatal MRI and toddler-age assessment of motor development have limited predictive value for school-age outcomes. These results reinforce the importance of serial assessment in children treated with cooling beyond 2 years to at least school age, even in those with developmental scores in the typical range at age 2 years.

It is important to note that, in this study, we did not limit our knowledge exclusively to the children's performance in standardized tests, and we also looked for parents' and teachers' perceptions of children's difficulties in daily life (an important and irreplaceable evaluation when the neurologic outcome is being measured). Curiously, we found that parents and teachers of children with HIE perceived their children's problems in a similar way as on the standardized tests, revealing a good awareness of the problem. However, it was very interesting that parents of children with HIE did not rate their children higher on the inattention scale than parents of children in the comparison group, advocating that some of the difficulties reported may only be observed in specific daily activities settings - at school by teachers.

CHARACTERIZATION OF A PRECLINICAL MODEL OF ENCEPHALOPATHY OF PREMATURITY BASED ON A PRENATAL ISCHEMIC INSULT

After these clinical studies, we decided to try to understand some of the mechanisms that could potentially explain the deleterious effects of ischemia in the neonatal brain. Performing an experimental study, we evaluated whether the neurodevelopmental, neurologic and behavioral outcomes would differ between a model of preterm birth induced by a non-inflammatory agent (mifepristone) and an inflammatory model of prenatal transient hypoxia-ischemia. Interestingly, we found that the late preterm (embryonic day 21) rats prenatally exposed to a non-inflammatory environment did not show significant alterations in the neonatal developmental milestones, anxiety, cognitive functions and neurochemical parameters. Conversely, hypoxia-ischemia rats showed delayed development of neonatal reflexes, reduction of anxiety, impairment of learning and memory, and alterations in neurogenesis, NPY-ergic and cholinergic systems. We concluded that prematurity by itself does not impact the brain directly, whereas prenatal hypoxia-ischemia clearly modifies developmental trajectory, learning and memory, neurogenesis, and NPY GABAergic and cholinergic brain systems. Late preterm birth, in a prenatal non-inflammatory environment, does not seem to constitute a handicap for neurologic development (at least in the neurological functions we evaluated) and it appears necessary to have an additional external insult, like hypoxia-ischemia, to induce major damage to the brain. Analogously to what happens in rats, we speculate that neurologic impairment observed in preterm children may result from consecutive exposure to environmental insults during the perinatal period and not from premature delivery itself or the absence of an intrauterine environment. In humans, this hypothesis is supported by the fact that preterm neonates with hypoxia–ischemia related risk factors (acidemia, low Apgar scores, apnoea, respiratory distress syndrome and seizures) are at increased risk of PVL and worse neurologic outcome[88]. Also, preterm neonates exposed to hypotension are at increased risk of developing encephalopathy of prematurity[89]. The hypothesis of an external insult to induce brain lesion also matches with the progressively better neurologic outcome of preterm infants that have been observed in the last decades, in parallel with improved care in neonatal units and reduced exposure of preterm neonates to environmental and physiologic stressors[90].

We observed that transient systemic hypoxia-ischemia (TSHI) exposed rats exhibited decreased anxiety levels and engaged in risky behavior in the elevated plus maze test and open field. This kind of behavior in rats, associated with their less interest in novelty, possibly due to attention problems, recapitulates the core symptoms of the attention deficit hyperactivity disorder commonly found in preterm children[91]. These results pointed ischemia as a possible cause of attention deficit hyperactivity disorder in preterm children and allow this experimental model to be used, in the future, to explore the pathophysiology of this association and look for new treatments.

In our work, we also demonstrated an impairment of learning and memory in TSHI rats. Gathering our results with information from previous studies, we verified that increasing the time of hypoxia-ischemia results in an increase in the severity of impairment in learning and memory[92,93]. Shorter periods of hypoxia-ischemia tend to affect learning first, and long periods affect learning and memory. Moreover, we showed TSHI rats had increased aberrant postnatal hippocampal neurogenesis. Because postnatal neurogenesis in the hippocampus is believed to contribute to learning and memory function, it is possible that the impaired spatial learning and memory observed in TSHI rats might be explained, at least in part, by the altered neurogenesis and aberrant migration of new neurons in the dentate gyrus[94]. These results provide a new mechanism by which exposure to ischemia may lead to long-term memory adverse outcomes and are in accordance with recent literature reporting the importance of adult neurogenesis in consolidating memories during sleep in mice[95]. Neurogenesis impairment may be one of the mechanisms involved in the learning and memory deficits recognized in preterm children, namely those exposed to ischemic insults.

Furthermore, in TSHI rats, the total number of NPY neurons was significantly increased in the hilus of the dentate gyrus. Given the neurotrophic effects of NPY[96] and our aforementioned results about neurogenesis, this could denote an NPY-dependent pathway by which ischemia would lead to increased neurogenesis, possibly via Y1 receptor[97]. It also highlights the role of NPY in response to hypoxia-ischemia and its potential role in treatment. However, despite the increase in the number of NPY neurons and neurogenesis, this was not enough to compensate for the damage induced by prenatal hypoxia–ischemia.

Moreover, we found that the density of the cholinergic varicosities was significantly reduced in the dentate hilus of TSHI rats and this might reflect dysfunction of the cholinergic basal forebrain since this region give rise to the majority of cholinergic projections to the

hippocampus[98]. Corroborating this hypothesis, cholinergic receptor blockade by scopolamine and mecamylamine exacerbates memory dysfunction induced by global cerebral ischemia in mice[99]. Impaired cholinergic basal forebrain integrity was already associated with cognitive deficits in a previous study where adults born preterm with cognitive deficits had smaller cholinergic basal forebrain volumes[100]. Therefore, our results provide supplementary evidence that cognitive deficits found in preterm children may be secondary to the ischemia effects in the cholinergic basal forebrain.

POTENTIAL ADDITIONAL DRUG TREATMENT FOR NEONATAL HYPOXIA-ISCHEMIA

Finally, after having a general overview of the effects of hypoxia-ischemia in the neonatal brain, through both a clinical and experimental perspective, we tried to go further and explore potential new treatments. Among the several potential ways of treatment, we started with a pharmacologic strategy. We tested three repurposed drugs (edaravone, perampanel, and metformin) by evaluating whether they can revert, or at least attenuate, the deleterious effects of hypoxia–ischemia in an in vitro model. We have found that edaravone and low concentrations of perampanel were able to attenuate cell damage induced by hypoxia and oxygen-glucose deprivation. Edaravone induced a decrease in reactive oxygen species and was the most efficient and the safest in the attenuation of events induced by hypoxia–ischemia. Our data corroborate previous reports that have demonstrated that edaravone increases cell viability and has neuroprotective properties[101,102]. In some hypoxic-ischemic models, edaravone attenuates hypoxia-induced hippocampal damage and cognitive impairment[103] however, in others edaravone was neuroprotective only to the acute phase[104] or did not improve neurological outcome at all[105]. The potential benefits of edaravone detected in cell lines are not so clear in animals subjected to ischemia. This fact could be related to several reasons including the dosage, interval, and route of drug administration. Nevertheless, our study reinforces the evaravone potential benefit in hippocampal cells, which are known to be important players in memory and other cognitive functions in humans and are frequently compromised by hypoxia-ischemic events.

We have found that perampanel, an antagonist of AMPA receptors, in low concentrations, exerts beneficial effects against hypoxia-ischemia. Although not so effective as edaravone in hippocampal cell lines, perampanel is capable of reducing neuronal overexcitation and could be very important from a clinical point of view. Due to its anti-seizure effect, perampanel could be an excellent candidate to be used in cases of neonatal HIE. HIE is often accompanied by seizures in the acute phase and currently the first-line medication, phenobarbital, has deleterious effects on neuronal cell survival[106]. Perampanel could be a good alternative antiseizure medication, with the advantage of having positive effects on cell survival in HIE.

THE ISCHEMIA PHENOMENA

Ischemia phenomena can affect the brain either focally, when it results from an occlusion of a blood vessel as in stroke, or diffusely when it results from a more global deprivation of oxygen and energy, as it occurs in HIE or encephalopathy of prematurity and PVL[13,37,107]. In our work, involving the cohort of children with neonatal HIE and in the experimental study involving the experimental model of prenatal ischemia, we confirmed that ischemia tends to affect grey matter regions such as thalamic nuclei, basal ganglia and hippocampus. Indeed, in our experimental study, we confirmed that the hippocampus is highly susceptible to hypoxia-ischemia events, affecting neurogenesis, GABA-ergic and cholinergic systems. Additionally, in the clinical review of neonatal stroke as well as in the HIE cohort, we have substantiated, once again, the susceptibility of grey matter, as children present mostly with seizures (an indirect sign of cortical involvement). MRI imaging data of our studies also support the involvement of the grey matter since in our HIE study involving a group of 23 children with full information about neonatal MRI, 13 children had signal abnormalities in the thalamus, 12 in the putamen, 10 in the globus pallidus, 7 in the hippocampus, and 3 in the caudate nucleus. Moreover, in the neonatal stroke study, MRI also demonstrated the involvement of the cortex and basal ganglia.

In preterm brain injury, it is well known that the white matter is greatly affected, as observed in preterm children with neonatal PVL[34]. In PVL, unlike stroke or HIE where neonates present with seizures or encephalopathy, acute neonatal clinical presentation tends to be subtler and

is often unrecognized. Despite the predominance of white mater injury typically observed in PVL, children with PVL and a higher number of ischemic risk factors (in which we assume a more severe ischemic insult) tend to have more often epilepsy (a sign of cortical involvement) compared to children with PVL and a higher number of infection-related risk factors. This favors again the cortical involvement in ischemia, even in a situation where white matter injury prevails.

It is known that infection is intrinsically related to ischemia in the neonate, and neonatal ischemic associated conditions share clinical risk factors associated with infection[6]. We confirmed in our reviews about neonatal stroke and PVL, that risk factors for these two ischemic diseases include risk factors associated with infection: intrapartum fever >38ºC, chorioamnionitis, neonatal sepsis and placental findings suggestive of infection. Exposure to infection exacerbates neuronal hypoxic-ischemic damage, leading to a phenomenon called infection-sensitised hypoxic-ischemic brain injury[6]. Given this intrinsic relation, inflammation could be viewed as the final pathway to these two triggers of injury. Perinatal infection (e.g., chorioamnionitis), hypoxia-ischemia, and other postnatal injurious triggers (e.g., mechanical ventilation) cause a self-perpetuating cascade of cerebral inflammation leading to white and grey matter lesions that ultimately underlie the spectrum of deficits found in children[108]. In our experimental work, we tested an inflammatory model induced by prenatal hypoxia-ischemia and compared it with a non-inflammatory model, where injury would be expected to be induced by preterm birth *per se*, with premature exposure of the neonatal brain to an extrauterine environment. However, our results showed that the non-inflammatory model does not impair postnatal neurodevelopment nor anxiety and cognitive functions in adolescent rats, which does not support the concept of dysmaturation of the nervous system induced prematurity but, instead, corroborates the view of a benign course of maturation of the nervous system in a non-inflammatory environment. Besides inflammation, data from our in vitro study, also reinforced the role of reactive oxygen species and excitotoxicity in the etiology of brain injury induced by hypoxia-ischemia, since we demonstrated the attenuation of the ischemic injury to neuronal hippocampal cells by blocking AMPA receptors and using the antioxidant properties of edaravone.

THE ISCHEMIA CONSEQUENCES

Regarding the clinical consequences of ischemia, from a didactic point of view, they can be divided into motor and non-motor impairments.

Motor impairment

We found that cerebral palsy, the severe presentation of motor impairment, occurred in about 70% of children with PVL and was present in 16% of the initial HIE cohort (before the application of the exclusion criteria cerebral palsy). Moreover, in the survey of children with neonatal stroke, 41% had hemiparesis. These results are in accordance with the existing medical literature[80,83]. Less severe motor impairment has also been noted after neonatal brain ischemia. When we looked for the motor consequences beyond cerebral palsy, we found that about one-fifth of children with HIE treated with TH had significant later motor impairment at school age, but these children with less severe motor dysfunction were not consistently identified by neurodevelopmental assessment at 2 years of age nor neonatal MRI. Mild motor impairment may pass unnoticed in the first years and only become visible some years later. From a clinical point of view, it is important to know how to easily identify these children in clinical practice in order to offer them special educational support, thereby allowing them a better academic outcome. Touwen neurological examination can be used for that purpose, as it allows the identification of children with MND, a condition we have found to be correlated with motor impairment and cognitive difficulties. Interestingly, in our work using the prenatal transient hypoxia–ischemia model we have found that albeit there was a clear impact on the developmental milestones and reflex development in pups in the first days (postnatal days 6 to 8), this effect was attenuated at postnatal day 9 to 10. However, even without significant impairment in developmental milestones and reflex development 10 days after birth, we observed clear alterations in the brain of the same rats later in the juvenile period, including impact on learning and memory and anxiety levels. Taken together, our experimental and clinical findings suggest that more attention is needed to the follow-up of children with a history of HIE, even if they do not show major motor impairments in the first period of life, because children may have subtle brain changes that only become clinically significant later (at school age) when the cognitive demands increase.

Non-motor impairment

The non-motor impairments associated with ischemia are well known[17] and were also naturally recognized in our clinical and experimental works. HIE can affect cognition and behavior, impairing learning, memory, language, attention and anxiety. As stated before, in our work on perinatal stroke, an abnormal neurodevelopmental assessment was found in 64% of children. Also, three-quarters of children with PVL had an abnormal developmental outcome, and most children evaluated with the Griffiths scale scored below average. Indeed, in rats, we showed that prenatal hypoxia-ischemia significantly affects learning, memory and attention. In an attempt to understand the neural substrates of these cognitive deficits, we investigated the hippocampus and we proposed that altered neurogenesis and aberrant migration of new neurons in this region could eventually explain memory deficits triggered by ischemia. Also, we have found that prenatal hypoxia-ischemia impacts the cholinergic system of the hippocampus and we speculate that the cholinergic system of the frontal cortex could be involved in cognitive dysfunction induced by ischemia in children born preterm. This explanation is similar to what has been proposed for the decline of cognitive function in vascular dementia[109].

Finally, we added evidence to the medical literature of the impairment of attention as a result of exposure of the neonatal brain to hypoxia-ischemia. In the cohort of children with neonatal HIE, attention was impaired when evaluated at school age, and, interestingly, inattention was correlated with poorer motor function. Moreover, in our experimental study, we observed that rats prenatally exposed to hypoxia-ischemia, mimic the core symptoms of human attention deficit hyperactivity disorder. Attention deficit hyperactivity disorder is a condition that has been associated with human ischemic brain disorders[110]. As an example, children with perinatal stroke are at increased risk of attention deficit hyperactivity disorder[111].

Motor and non-motor impairments are intrinsically linked, seems to develop in parallel and potentiate each other. In case of motor impairment, the non-motor functions can be indirectly affected.

LIMITATIONS OF THE RESEARCH

The main limitations of each study were already addressed in the discussion section of each study. From a global perspective, the clinical studies included in the thesis have a small number of patients and that condition limited our conclusions and imposed precautions in the extrapolation of the results. The experimental study in rodents would have benefited from the histological analysis of other brain regions besides the hippocampus, to have a more comprehensive vision of the effects of ischemia in the neonatal brain. Furthermore, the in vitro study did not include a neuronal progenitor cell line which would be interesting, given the potential role of neurogenesis in the pathogenesis of neurologic deficits and neuronal repair.

A FINAL REFLECTION

Despite enormous advances in neonatal care to prevent neonatal brain injury and the associated neurodevelopmental impairment, currently, many neonates grow into adulthood with important and incapacitating handicaps. Predicting long-term outcomes in neonates remains a difficult adventure, but improved and specific clinical and ancillary evaluation could facilitate the task. Parents of neonates admitted to neonatal intensive care with brain injury inevitably face many doubts, afflictions, and distress. Estimating the prognosis is essential to adequately inform the child's family and begin intervention therapy. Brain plasticity is highest in the first period of life and we need to take that advantage to implement suitable therapies as soon as possible. An individualized approach to neonatal brain injury and neurologic-oriented precision therapy is warranted.

We have reviewed the most common perinatal clinical conditions leading to ischemic brain injury. We analysed the outcome of children with PVL and the relation with prenatal exposition to ischemia and infection. We reviewed the outcome of perinatal arterial ischemic stroke in a small series of patients in an attempt to correlate it with identified risk factors. We reported the clinical outcome of children who suffered neonatal HIE, but not developed cerebral palsy, and highlighted the importance of MND. We characterized a preclinical model of encephalopathy of prematurity based on a prenatal ischemic insult and postulated some

explanations for neurologic deficits. Finally, three drugs were tested in an in vitro model trying to select a drug to ameliorate neurologic deficits associated with ischemia. Translation of the knowledge learned from experimental work to humans was considered and discussed.

All these perinatal conditions share some common pathophysiologic aspects (hypoxia-ischemia leading to inflammation, oxidative stress, excitotoxicity...) and outcome (motor and non-motor deficits). All embrace a road of life-long disabilities varying from severe cerebral palsy to MND but also exists the possibility of a normal outcome. It is our job to improve that possibility!

IMPORTANT CLINICAL IMPLICATIONS OF THIS THESIS

- Inflammation triggered by hypoxia-ischemia or infection should be avoided at all costs to improve the neurologic outcome of neonates admitted to neonatal intensive care units
- A non-inflammatory prenatal environment is safe for the neonatal brain and should be promoted in prenatal medical appointments
- The pathophysiology of some cognitive deficits found in preterms, namely memory, learning and attention, are now better understood
- A long-term follow-up of children exposed to hypoxia and ischemia in the perinatal period is needed, even if the first years of life show a normal development
- MND in children after neonatal HIE should not be overlooked as it may predict mild motor impairment and learning difficulties at school age. Touwen neurological exam may help to identify children with these difficulties
- Perampanel should be explored as an alternative to phenobarbital in case of seizures in HIE. In contrast to phenobarbital, perampanel has a positive effect on hippocampal cell survival. Further animal research is needed to test this hypothesis.

FUTURE PERSPECTIVES

Future investigation in this field should include identifying genetic factors involved in the susceptibility of ischemic brain injury. It would also be interesting to identify better biomarkers of hypoxia-ischemia in preterms in order to further understand the magnitude of the problem in these neonates. Test perampanel and edaravone in neuronal progenitor cells may help to clarify their role in the protection against hypoxia-ischemia injury and, from a clinical point of view, it would be important to elucidate the role of perampanel in neonatal seizures under hypoxic-ischemic conditions.

CONCLUSION

- Children with PVL who were perinatally exposed to infection may have a higher risk of abnormal developmental outcome or cerebral palsy compared with children with PVL and perinatally exposed to hypoxia-ischemia. This could reflect a more severe white matter injury induced by inflammation associated with infection. Children exposed to ischemia had more epilepsy and hearing impairment.
- Children with ischemic perinatal stroke had at least one risk factor and the presence of multiple risk factors was significantly associated with worse outcome. The combination of various risk factors may contribute to a more severe brain injury.
- In the absence of cerebral palsy, about one-fifth of children with HIE treated with TH had significant motor impairment at school age. Motor development is intrinsically linked to cognitive development and attention capacity may contribute to motor performance. Early neurodevelopmental assessments are poor predictors of mild motor impairment. Long-term follow-up of children with perinatal HIE is essential.
- Prenatal hypoxia–ischemia delays neonatal developmental milestones and impairs learning, working and long-term memory, as well hippocampal postnatal neurogenesis, cholinergic innervation and NPY expression. Disrupted postnatal hippocampal neurogenesis could explain memory deficits found in preclinical and clinical settings and reduction in cholinergic innervation of the hippocampus constitute one possible explanation for cognitive deficits. Exposure to the extrauterine environment before term age in the absence of inflammation (e.g., hypoxia-ischemia) does not seem to grossly impact the normal neurologic outcome.
- Edaravone and perampanel at low concentrations can attenuate cell damage induced by hypoxia and oxygen-glucose deprivation.

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