A CLINICALLY RELEVANT MODEL AND A POTENTIAL TREATMENT OF PERINATAL HYPOXIC ISCHAEMIC ENCEPHALOPATHY IN MICE

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I. Abstract

Hypoxia ischaemia (HI) due to neonatal asphyxia is the most common cause of acute mortality and chronic neurological disability. Currently there is no effective means to repair the damaged brain. The aim of this study was to 1) establish a clinically relevant intrauterine model of perinatal hypoxic ischaemic encephalopathy (HIE) in mice, 2) determine whether lipopolysaccharide (LPS) induced maternal systemic inflammation worsened the outcome of neonates who had previously suffered HIE brain damage and 3) use the mouse HIE model to study whether a docosahexaenoic acid (DHA) enriched maternal diet has the potential to alleviate this harmful clinical condition.

This is the first time that the intrauterine HI model has been established in mice. Pups exposed to 15 minutes of HI showed a higher mortality rate at 1 hour and those who survived displayed worse short term outcomes. However, the results were inconsistent with regards to the cellular changes observed such as inflammation and apoptosis, histological evidence and long-term neurocognitive outcomes. LPS-induced maternal systemic inflammation combined with HI showed no difference in survival rates compared to HI alone with a lower hypoxia-inducible factor 1-alpha and higher phospho-p38 and phospho-bad levels in the pup brains being observed. A maternal diet enriched with DHA did not result in a better outcome in the DHA + HI pups. The greater mortality rates in both Caesarean section (CS) and HI groups regardless of DHA in the diet indicated that other factors may influence neonatal death rates.

Based on these data, this study concludes that the 15 minutes intrauterine HIE model cannot provide sustained damage observed in the mice brain and that the maternal LPS induced systemic inflammation did not show a clear better or worse outcome in the offspring. In addition, no clear neuroprotective effect was seen for the DHA enriched diet group.

II. Declaration of Originality

I hereby confirm that all of the work in this thesis is my own. Where all else has been obtained from others is appropriately referenced in the text.

Xia Zheng

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III. Copyright Declaration

IV. Acknowledgements

I would like to take this opportunity to extend my sincere gratitude to the people who have made my PhD possible.

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V. Table of Contents

VI. List of Figures

Chapter 5

VII. List of Tables

VIII. Abbreviations

AA	Arachidonic acid
ADP	Adenosine diphosphate
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
Apaf1	Apoptotic protein activating factor 1
ATP	Adenosine triphosphate
AUC	Area under curve
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
BHT	Butylated hydroxyl toluene
BSU	Biological Services Unit, University College London
CAD	Caspase activating DNAse
CBS	Central Biomedical Services, Imperial College London
CCL	Chemokine (C-C motif) ligand
CNS	Central nervous system
CS	Caesarean section

CXCL	Chemokine (C-X-C motif) ligand
Cyt C	Cytochrome C
DAB	3, 3 -diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EAAT	Excitatory amino acid transporter
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acids
EPO	Erythropoietin
ERK	Extracellular signal-regulated kinases
FADD	Fas adaptor death domain protein
FAMEs	Fatty acid methyl esters
FASL	Fas death receptor ligand
FI	Fluorescence intensity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography

G-CSF	Granulocyte-colony stimulating factor
GFAP	Glial fibrillary acidic protein
Gln	Glutamine
Glu	Glutamate
H&E	Haematoxylin and Eosin
HI	Hypoxia ischaemia
HIE	Hypoxic Ischaemic Encephalopathy
HIF-1α	Hypoxia-inducible factor 1-alpha
HO-1	Heme oxygenase-1
IAP	Inhibitor of apoptosis
IFN-γ	Interferon gamma
IL	Interleukin
LA	Linoleic acid
LPS	Lipopolysaccharide
mRNA	Messenger RNA
n-3 PUFAs	Omega-3 polyunsaturated fatty acids
n-6 PUFAs	Omega-6 polyunsaturated fatty acids
NF-κB	Nuclear factor kappa B

NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOR	Novel object recognition
NPD1	Neuroprotectin D1
Nrf2	E2-related factor 2
OFT	Open field test
PARP1	Poly-ADP-ribose polymerase 1
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween® 20
PCr/Pi	Phosphocreatine/inorganic phosphate
PFA	Paraformaldehyde
PGHS-2	Prostaglandin G/H synthase 2
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red blood cell
RM1	Rat and Mouse No.1 maintenance autoclavable diet
RNA	Ribonucleic acid
SD	Standard deviation

SDS	Special Diets Services
SEM	Standard error of the mean
SPM	Specialised pro-resolving mediator
STAT3	Signal transducer and activator of transcription 3
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween® 20
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TUNEL	Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling
VDCC	Voltage-dependent calcium channels
WM	Water maze

Chapter 1. Introduction

1.1 Hypoxic ischaemic encephalopathy (HIE)

Perinatal asphyxia, more appropriately known as hypoxic ischaemic encephalopathy (HIE), is one of the most common causes of neonatal death and long-term neurological sequelae. It occurs when the brain is deprived of oxygen during the birth process.

1.1.1 Definitions

In the past, a number of definitions have been used in published works to describe conditions that affect neonates and these include (but are not limited to): postanoxic encephalopathy [8], HIE [9], post-asphyxial encephalopathy [10], birth asphyxia [11], intrapartum fetal asphyxia [12] and neonatal encephalopathy [13-15]. These terms were used based on different combinations of criteria, for instance, conscious level, respiration, tone, posture and reflexes. Most studies describe these conditions by using the Sarnat and Sarnat criteria which are shown in Table 1.1 [8]. HIE can be a diagnosis based on a history of asphyxia that has caused acidaemia, a low Apgar score, neurological damage and the involvement of other organs such as the heart, lungs, livers and kidneys. Apgar scores were first described by Virginia Apgar in 1952. It is a method to quickly evaluate the newborn baby's health by five simple criteria: appearance (skin colour), pulse (heart rate), grimace (reflex irritability), activity and respiration. The resulting Apgar score ranges from zero to ten and the criteria are shown in Table 1.2. A clinical grading system for HIE was introduced by Sarnat and Sarnat [8] to describe the neurological abnormalities which were subsequently modified by Levene *et al* [7]. According to the grading system, HIE can be further classified into 3 grades: mild, moderate and severe (Table 1.3). Studies have reported that the outcomes of infants who suffered mild HIE were comparable to those of normal full term infants while infants who suffered severe HIE had much poorer outcomes and may either die or developed cerebral palsy or other cognitive deficits [16, 17].

Table 1.1 The original Sarnat & Sarnat criteria for the measurement of HIE

Level of consciousness	Stage 1 (mild) Hyperalert	Stage 2 (moderate) Lethargic/obtunded	Stage 3 (severe) Stuporous
Neuromuscular control			
Muscular tone	Normal	Mild hypotonia	Flaccid
Posture	Mild distal flexion	Strong distal flexion	Intermittent decerebration
Stretch	Overactive	Overactive	Decreased/absent
Segmental myoclonus	Present	Present	Absent
Complex reflexes			
Suck	Weak	Weak/absent	Absent
Moro	Strong	Weak	Absent
Oculovestibular	Normal	Overactive	Weak/absent
Tonic neck	Slight	Strong	Absent
Autonomic function			
Pupils	Mydriasis	Miosis	Variable
Heart rate	Tachycardia	Bradycardia	Variable
Bronchial/salivary secretions	Sparse	Profuse	Variable
Gastrointestinal motility	Normal/decreased	Increased/diarrhea	Variable
Seizures	None	Common/focal or multifocal	Uncommon
EEG	Normal/decreased	Early low voltage continuous delta and theta, later periodic, seizues focal 1–1.5 Hz spike-wave	Early periodic pattern with isopotential phases, later isopotential
Duration	<24 h	2–14 days	Hours–weeks

EEG, electroencephalogram.

*Table 1.1 is adapted from Lee *et al* [18].

Table 1.2 The five criteria for Apgar scores

	Score of 0	Score of 1	Score of 2
Appearance (skin colour)	Blue or pale all over	Blue at extremities body pink	No cyanosis body and extremities pink
Pulse (heart rate)	Absent	< 100 beats per minute	> 100 beats per minute
Grimace (Reflex irritability)	No response to stimulation	Grimace on suction or aggressive stimulation	Cry on stimulation
Activity	None	Some flexion	Flexed arms and legs that resist extension
Respiration	Absent	Weak, irregular, gasping	Strong, lusty cry

Table 1.3 Clinical grading system for HIE adapted from Levene *et al* [7]

Grade 1 (mild)	Grade 2 (moderate)	Grade 3 (severe)
Irritability 'hyperalert'	Lethargy	Comatose
Mild hypotonia	Marked abnormalities in tone	Severe hypotonia
Poor sucking	Requires tube feeds	Failure to maintain spontaneous respiration

There are various pathological signs that appear in the brain following HIE. The pattern of injury depends on the severity of the insult, the timing and duration and the maturity of the brain. Clinically, observed patterns after HIE injury include cerebral oedema, selective neuronal necrosis, basal ganglia and brain stem injury, parasagittal injury, white matter injury and focal cerebral infarction, with selective neuronal necrosis being the most commonly observed [19]. Magnetic resonance imaging (MRI) has markedly improved the understanding of the patterns of brain injury of HIE. A cohort study, with 173 term newborn babies with a clinical diagnosis of HIE, showed that 45% had watershed MRI patterns while 25% had basal ganglia/thalamus patterns and the basal ganglia/thalamus patterns were associated with more severe neonatal signs of damage [20].

1.1.2 Incidence

In the past 30 years, the incidence of HIE is estimated to be 1-4 per 1,000 live term births in developed countries [15, 21-24]. However, in developing countries, the number is higher; for example, in China it is around 3-6 per 1,000 live term births [25]. In one study from Africa, the incident of HIE is ranged from 8.5 to 13.3 per 1,000 live births according to different

criteria of HIE [26]. Among survivors, cerebral palsy, functional disability and cognitive impairment often develop later in childhood. Neonates may develop multiorgan failure as a result of HIE which is responsible for the majority of deaths during the neonatal period. Data from India show that HIE contributes to 16% of neonatal death [27]. About one third of the survivors will go on to develop a significant neurodevelopmental disability, most commonly cerebral palsy, but also epilepsy, mental retardation and learning disabilities [28]. There is no doubt that HIE has an enormous cost and heavy burden of care for patients' families. It is disappointing to note that currently no treatment is effective.

1.1.3 Risk factors

There are very few papers which deal with the risk factors associated with HIE. This is partly due to the fact that there is no universal agreement concerning the definition of HIE and also the big differences for the incidence of HIE as previously discussed. Risk factors of HIE may be both maternal and fetal. The fetal risk factors include, but are not limited to, multiple pregnancy, chromosomal anomaly, fetal growth restriction and prematurity [29]. The maternal risk factors can be classified into antepartum and intrapartum risk factors based on the time that the fetal brain is exposed to insufficient supply of oxygen. In general, the antepartum risk factors include maternal hypotension, pre-eclampsia, bleeding, infection and thyroid disease; while the intrapartum risk factors include forceps delivery, infection, prolapse of the cord, placental abruption, uterine rupture, the absence of electronic fetal mornitoring, home births and water births [15, 30, 31].

In developing countries, maternal deficiency states is one of the major antepartum factors for HIE. A study carried out in Nepal has shown that high maternal age (>35 years), primiparity, short stature (<145 cm) and not having antenatal care are the significant independent preconceptual and antepartum risk factors [32]. This study also suggest that prolonged

rupture of membranes and the use of oxytocin are the most important intrapartum risk factors for HIE.

In developed countries, the risk factors for HIE are slightly different from those in developing countries. Researchers in Australia found that intrauterine growth restriction, high maternal age and some social and demographic factors such as, unemployment and being a housewife, increase the risk for HIE [30]. Results from the same team also revealed that although maternal pyrexia, persistent occipito-posterior position and acute intrapartum events are intrapartum risk factors for HIE, these account for only 4% of the risk for HIE [15, 33]. However, they found that it is interesting that both vaginal delivery and emergency caesarean section increased the risk for HIE while elective caesarean section has a reducing effect [15]. Another study in Sweden showed that leanness of the mother was a risk factor for HIE [34].

1.2 Mechanisms of HIE

The mechanisms of HIE are heterogeneous and many papers have discussed them. It has been generally considered that there are two parts to neuronal injuries when HIE has occurred. The brain injury begins during the primary neuronal insult and during the recovery processes are set in motion that cause secondary neuronal injury [35, 36]. The primary neuronal injury happens within 8 hours after the initial HI damage, then the neonates appears more active for 8 to 24 hours and after 24 hours, seizures and other clinic signs develop [37, 38]. Simplified schematic representations of the mechanisms of HIE are shown in Figs 1.1 A and B.

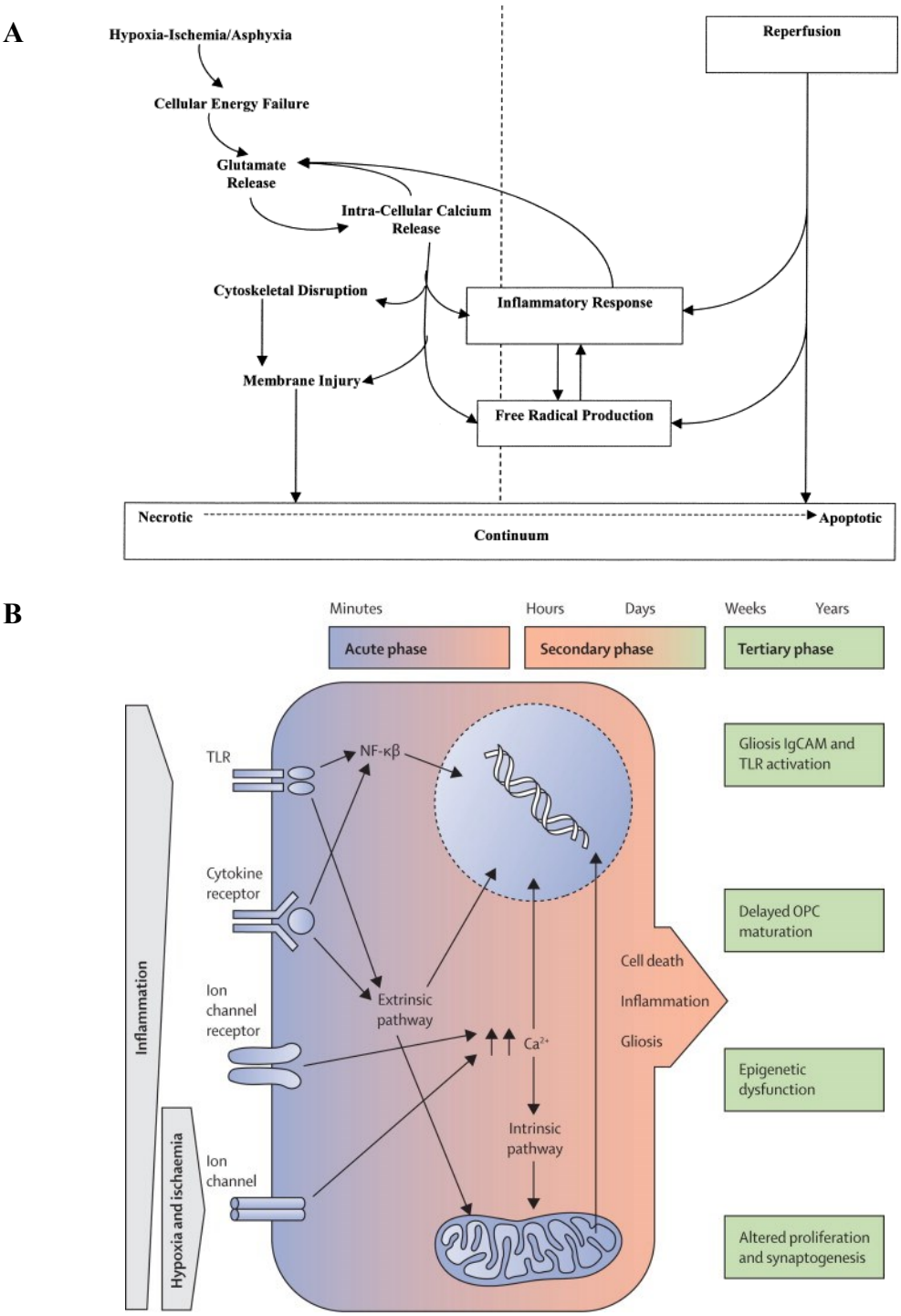


Fig 1.1. Diagrammatic representations of the mechanisms involved in HIE.
A Simplified diagram of the pathophysiologic mechanisms involved in HIE brain injury. Oxidative stress and excitotoxicity, through downstream intracellular signalling, produce both inflammation and repair. The cell-death phenotype changes from an early necrotic morphology to a pathology resembling apoptosis. **B** Outline of the primary, secondary and tertiary phases of HIE. Only selected tertiary phase mechanisms are shown and it is not being discussed in this Chapter. Cell death begins immediately and continues during a period of days to weeks. *Fig A is modified fromVannucci [1] and Bona *et al* [2]; Fig B is adapted from Fleiss and Gressens [5].

1.2.1 Primary neuronal injury

In this phase, the cellular hypoxia can lead to a cascade of biochemical events (Fig.1.2). Firstly, due to the lack of O_2, oxidative phosphorylation stops and leads to depletion of high-energy phosphate compounds (i.e. adenosine triphosphate (ATP) and phosphocreatine). As a result, glycolysis becomes the only source of ATP production in the brain. Compared with the efficient oxidative phosphorylation method of ATP generation (with 36 molecules of ATP generated per molecule of glucose consumed), glycolysis only produces 2 molecules of ATP per molecule of glucose. Glycolysis also causes lactic acid accumulation. This primary energy failure then causes neuronal membrane depolarization and membrane pumps (Na^+/K^+ adenosine triphosphatase and Na^+/Ca^{2+} exchanger) failure. Following failure of the membrane pumps, the accompanying influx of sodium ions (Na^+) and water leads to cytotoxic cerebral oedema and necrotic cell death. The cellular efflux of potassium ions (K^+) then induces a release of glutamate into the synaptic cleft and because of a failure of energy-dependent re-uptake, a massive accumulation of glutamate occurs [39].

Glutamate is the most common excitatory neurotransmitter found in the synapses in the brain and spinal cord [40]. An excess of glutamate exhibits neuronal toxicity and this condition is known as excitotoxicity [41]. Glutamate opens the N-methyl-D-aspartate (NMDA) receptor-channel complex leading to calcium (Ca^{2+}) influx into neuronal cells [42, 43]. Simultaneously, because of the Na^+/Ca^{2+} pump failure, intracellular Ca^{2+} is further increased. This massive accumulation of Ca^{2+} within the cell cytoplasm causes an increase of nitric oxide (NO) by activation of NO synthase. NO affects neurons adversely and leads to the formation of peroxynitrite, a toxic free-radical that is known to alter cell membranes by increasing the activity of Ca^{2+} ATPase leading to further intracellular accumulation of Ca^{2+} [44, 45]. Intracellular Ca^{2+} flooding also activates other enzymes, such as caspases, proteases, phospholipases and endonucleases. Free radicals and catabolic enzymes destroy cellular

membranes, nucleic acids, and other cellular contents, causing neuronal necrosis. The processes leading to necrosis occur within the first few minutes after ischemia and continue for a number of hours.

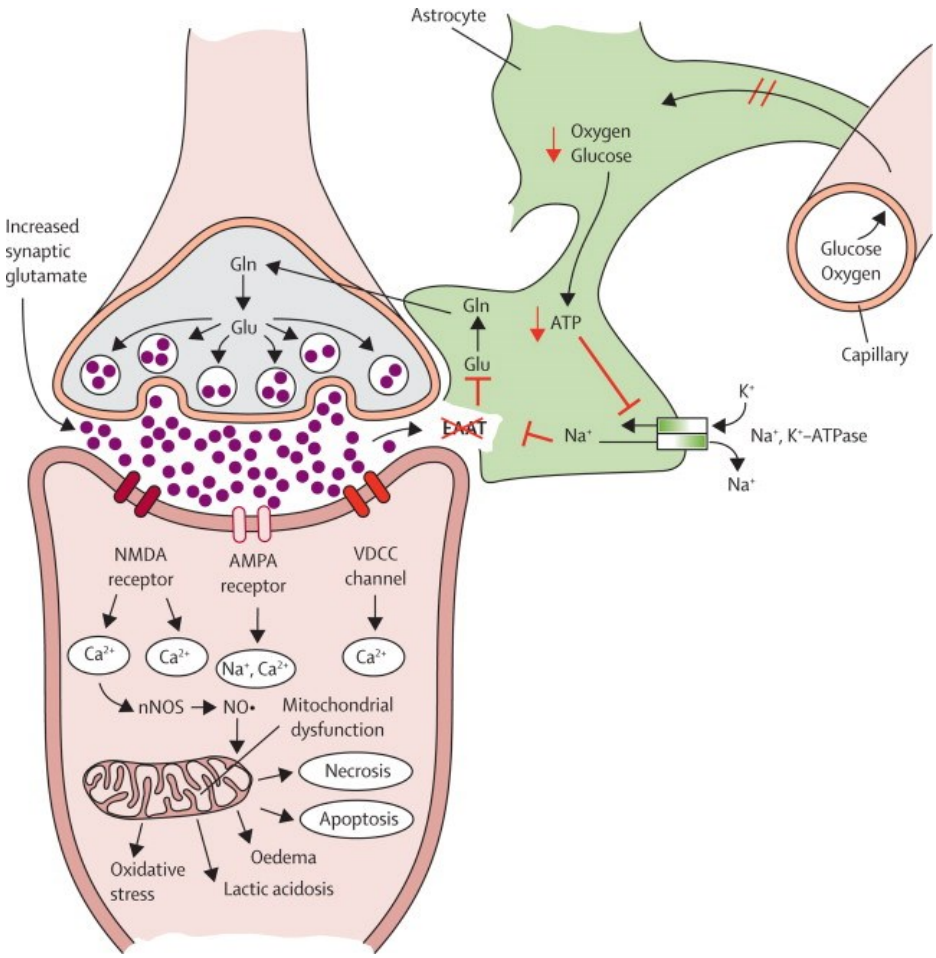


Figure 1.2. Excito-oxidative cascade of events that mediate hypoxic-ischaemic brain injury. Severe hypoxia impairs oxidative metabolism leading to neuronal depolarisation and ischaemia. Ischaemia reduces delivery of the glucose necessary for anaerobic metabolism and floods the synaptic cleft with glutamate and calcium. Subsequent activation of nitric oxide synthetase leads to high levels of nitric oxide. This toxic free radical attacks enzymes associated with oxidative phosphorylation and electron transport. Calcium attacks mitochondria and other cellular machinery. Signals released from damaged mitochondria lead to apoptosis. This excito-oxidative cascade occurs over a period of days to weeks. EAAT = excitatory amino acid transporter. Gln = glutamine. Glu = glutamate. nNOS = neuronal nitric oxide synthase. NO = nitric oxide. VDCC = voltage-dependent calcium channels. Adapted from Johnston *et al* [6].

1.2.2 Secondary neuronal injury

Following a short resuscitation and restoration period (approximately 30-60 minutes), the concentration of phosphorus metabolites and intracellular pH normalises with transient improvement of cytotoxic oedema [46]. The reperfusion causes further delayed injury through the inflammatory response at the site of injury.

A study using the asphyxiated new born piglet model showed that the secondary phase of injury lasts from hours to days and with a normal intracellular pH and stable cardiorespiratory status [47]. The cellular response during this phase of neuronal injury is similar to the primary phase of injury. This secondary phase is also characterized by a decrease in the ratio of phosphocreatine/inorganic phosphate (PCr/Pi) leading to a secondary energy failure [47]. Similar to the primary injury, the cascade of pathologic processes following reperfusion triggers accumulation of glutamate, increased Ca^{2+} accumulation, generation of free radicals and activation of phospholipases as well as other metabolic events leading to further neuronal cell injury. Increased intracellular Ca^{2+} also activates a cascade of proteolytic enzymes. These proteolytic enzymes, especially caspases or cystein proteases, will eventually trigger cellular nuclear fragmentation [37, 48, 49]. Additionally, inflammation plays a vital role in the accompanying oxidative stress and excitotoxicity [50]. As excitotoxicity has been discussed in the primary injury, the rest of the mechanisms involved in the secondary phase of injury would be discussed individually. Several pathways of the secondary injury are presented in Fig 1.3.

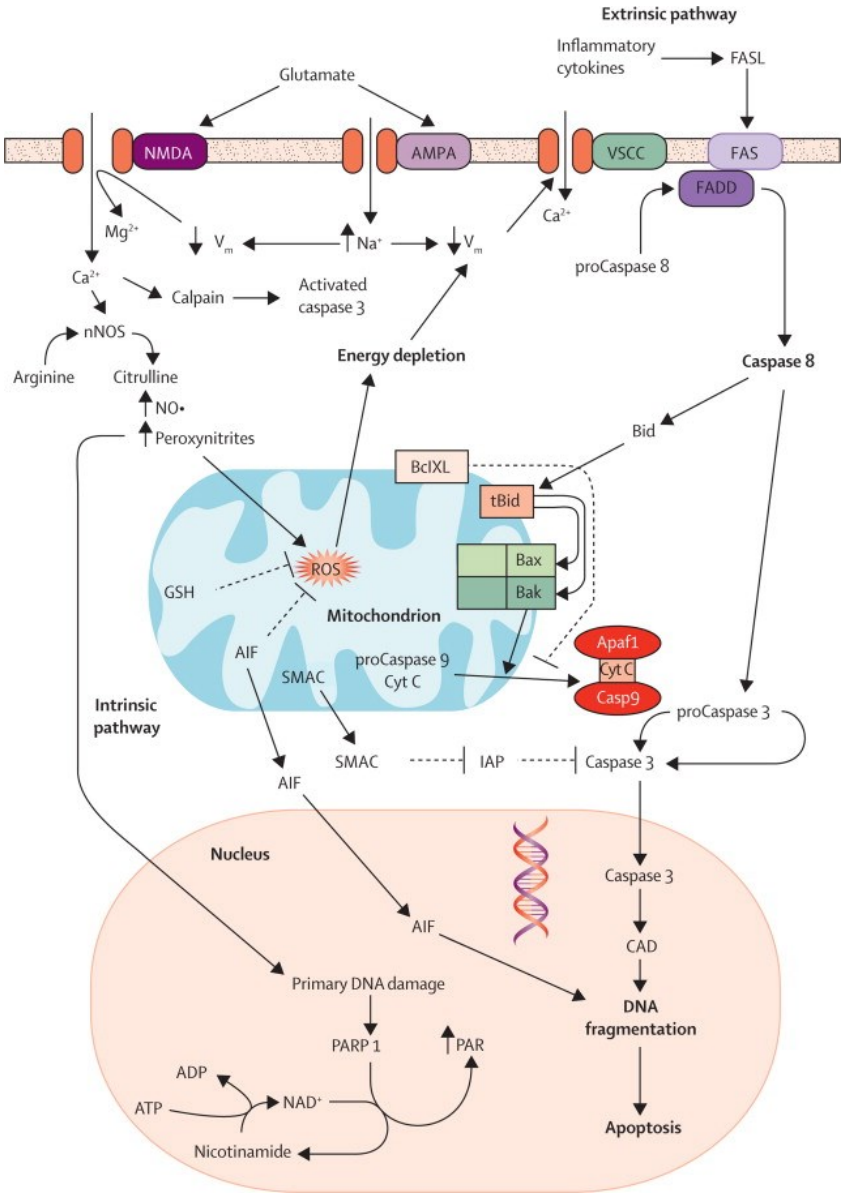


Figure 1.3. Downstream signalling pathways that mediate the apoptosis–necrosis continuum. Vm = membrane potential. VSSC = voltage sensitive calcium channel. Fas = death receptor in tumour necrosis factor family. FADD = fas adaptor death domain protein. Bcl-XL= anti-apoptotic proteins in the Bcl-2 family of proteins. Bax and Bak = pro-apoptotic proteins that form channels in outer mitochondrial membrane releasing cytochorome C to trigger apoptosis. tBid = truncated BH3-only pro-apoptotic protein. Apaf1 = apoptotic protein activating factor 1. IAP = inhibitor of apoptosis. CAD = caspase activating DNAse. Cyt C = cytochrome C. nNOS = neuronal nitric oxide synthase. NO = nitric oxide. ROS=reactive oxygen species. PARP1 = poly-ADP-ribose polymerase 1. PAR = poly-ADP ribose formed by ribosylation of DNA and proteins. GSH = glutathione, an antioxidant. AIF = apoptosis-inducing factor. Bid = BH3 interacting domain pro-apoptotic protein. NAD = nicotinamide adenine dinucleotide. FASL = Fas death receptor ligand. Data are adapted from Johnston *et al* [6]

1.2.2.1 Mitochondrial failure and apoptosis

As mentioned previously, the opening of NMDA channels lead to an influx of Ca^{2+} that causes NO synthase activation, which leads to production of free radicals such as NO and peroxynitrite. Toxic peroxynitrite molecules can add nitrates to tyrosine groups on proteins and contribute to the production of hydroxyl radicals, causing lipid peroxidation of proteins and DNA (Fig 1.3). During hypoxia, nitric oxide can also disrupt mitochondrial respiration by impairing the function of cytochrome oxidase, which increases production of superoxide and peroxynitrite ions within mitochondria [51, 52]. Accumulation of lactic acid can result in mitochondrial failure, and this can be measured with proton magnetic resonance spectroscopy during HIE progression [53]. These ions can enhance the movement of the pro-apoptotic proteins across the outer mitochondrial membrane into the cytoplasm where they trigger apoptosis through the intrinsic pathway. The B cell lymphoma 2 (Bcl-2) family of proteins plays an important role in cancer, cell death and neurodegeneration [54]. There are two main functional classes of proteins in the Bcl-2 family: pro-apoptotic proteins which can interact with other pro-apoptotic members to initiate cell death signalling or with anti-apoptotic Bcl-2 family proteins to inhibit cell death signalling (i.e. by inhibiting through proteins such as Bax) and anti-apoptotic members (i.e. through Bcl-2). Caspase-3 is a validated marker in detecting early neuronal apoptosis [55]. Bcl-2 has been found to act downstream from mitochondrial cytochrome C release thereby preventing the caspase-3 dependent proteolytic cascade which in turn protects the cell from apoptosis [56].

Hagberg *et al* reported that outer mitochondrial membrane permeabilisation in neonates was initiated by the pro-apoptotic protein Bax and regulated by the anti-apoptotic Bcl family proteins [50] (Fig 1.3). It has been also noted that a Bax-inhibiting peptide reduces neonatal brain injury and functional impairment in a mouse model of HIE [57, 58]. In the cytoplasm, cytochrome C binds with caspases in the apoptosome to trigger activation of caspase-3,

which in turn triggers apoptotic DNA fragmentation (Fig 1.3) [59]. Apoptosis-inducing factor then moves into the nucleus and triggers DNA fragmentation via a non-caspase-mediated mechanism that is stimulated by the DNA repair enzyme poly-ADP-ribose polymerase (PARP1) (Fig 1.3) [60].

A recent study also has shown that calcium entering through NMDA receptors can also activate caspase-3 directly without activating neuronal NO synthase through activation of calpain [61]. Caspase-3 is expressed to a much greater degree in the immature brain than in the adult brain which makes the neonatal brain more vulnerable to injury. Apoptosis can also be triggered by the Fas cell-death protein cell-surface receptor through activation of caspase-8 and then caspase-3 in the extrinsic cell-death pathway (Fig 1.3). It has been shown that transgenic mice that lack Fas receptors are resistant to hypoxic-ischaemic brain injury.

1.2.2.2 Inflammation

Inflammation also plays a role in the oxidative stress and excitotoxicity cascade during the perinatal period [50]. As previously mentioned, epidemiological studies have shown that identification of maternal infections during pregnancy is one of the risk factors for HIE [62-64]. Animal studies have shown that bacterial lipopolysaccharide (LPS) can sensitise the perinatal brain to HIE [65]. LPS is recognized by the toll-like receptors, in particular TLR4, [66], which leads to an inflammatory reaction involving oxygen free radicals and the synthesis of pro-inflammatory cytokines such as interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) [67]. Other studies also found that the majority of HI injury induced by LPS can be reduced by administration of the antioxidant N-acetylcysteine [68]. This effect is dependent on activation of microglia and up-regulation of inflammatory mediators via nuclear factor kappa B (NF-κB). Treatment with an inhibitor of NF-κB, after the onset of

neonatal hypoxia-ischaemia, has been shown to provide substantial protection against neonatal hypoxia-ischaemia by inhibiting apoptosis [69].

1.3 Therapeutic strategies

1.3.1 Current Therapeutic Strategies

Recently, many clinical trials have reported that whole body or head cooling for 72 hours at 33.5˚C can possibly reduce mortality and disability at 18-month follow-up in infants born with HIE [70-74]. As a result, the UK National Institute for Health and Clinical Excellence and the British Association of Perinatal Medicine have published guidelines for the National Health Service (NHS) to use hypothermia as a normal treatment for this condition [75, 76]. However, although these studies claimed that therapeutic hypothermia improves outcomes of the infants with HIE, 40-50% of the infants still die or have significant disability at 18 months [77]. Also, among these studies, the CoolCap Study Group trial suggested that head cooling had no effect in infants with severe HIE [73]. Another randomised trial using whole-body cooling reported that there was no significant reduction in the combined rate of death and severe disability [78]. Nevertheless, infants must meet certain criteria to be eligible for therapeutic hypothermia. Therefore, it is important to develop other possible ways to either protect the neonatal brain from HIE damage or cure the damaged brain.

1.3.2 Potential Therapeutic Strategies

Different potential strategies target that different mechanisms of HIE will be briefly discussed here.

1) Antiexcitotoxic agents are the earliest pharmacologic strategies to protect the neonatal brain. These agents include magnesium sulfate, a blocker of the NMDA receptor, which has have been shown to reduce moderate to severe HIE rate. However, it shows no significant reduction in mortality rate in survivors in large clinical trials [79]. Another potential anti-excitotoxic agent, xenon, will be discussed later.

2) Antioxidants and anti-inflammatory agents such as allopurinol, melatonin and vitamin E. Among these agents, melatonin has shown efficacy in brain protection against HIE in both animal [80] and human studies [81]. Another promising strategy is omega-3 polyunsaturated fatty acids (n-3 PUFAs), which will be discussed later.

3) Growth factors, such as erythropoietin (EPO), have been tested in animal studies and shown that it can improve the neurobehavioral outcomes after neonatal HIE [82, 83].

4) Other strategies such as hyperbaric oxygen [84] and stem cells [85] therapies are only considered to be potential treatments for HIE. Although hyperbaric oxygen has been used in China, it has not been accepted by the rest of the world. Thus, studies to prove the efficacy of both of these therapies are currently in progress.

1.3.2.1 Docosahexaenoic acid (DHA)

The brain grows rapidly during gestation and during the first twenty years after birth the brain more than triples in size [86-88]. DHA is one of the most important essential fatty acids and it is highly enriched in brain, nervous tissue and retina [89, 90]. Before birth, all of the n-3 PUFAs required for fetal development must be provided by placental transfer from the maternal circulation [91]. For the mothers, DHA can be obtained directly from the diet or by synthesis from its precursor alpha-linolenic acid (ALA), which is also an n-3 PUFA that can be found in seeds, nuts and many vegetable oils. In fact, the human body cannot synthesise

all the DHA necessary for optimum performance from ALA, needing to obtain it mostly from the diet [92]. The metabolic synthesis of DHA is carried out in the liver and this involves a series of alternating desaturation and elongation reactions [93, 94]. This process is shown in Fig 1.4. As seen in the figure, an omega-6 polyunsaturated acid (n-6 PUFA) and arachidonic acid (AA), share the same enzymes as DHA when metabolised from linoleic acid (LA) [95]. As a result, n-3 and n-6 PUFAs may compete with each other which may lead to varying levels in brain and other organs [96]. Studies have shown that high intake of LA inhibit the n-3 PUFA metabolism pathway and reduce the accumulation of DHA in brain and other organs [97-100].

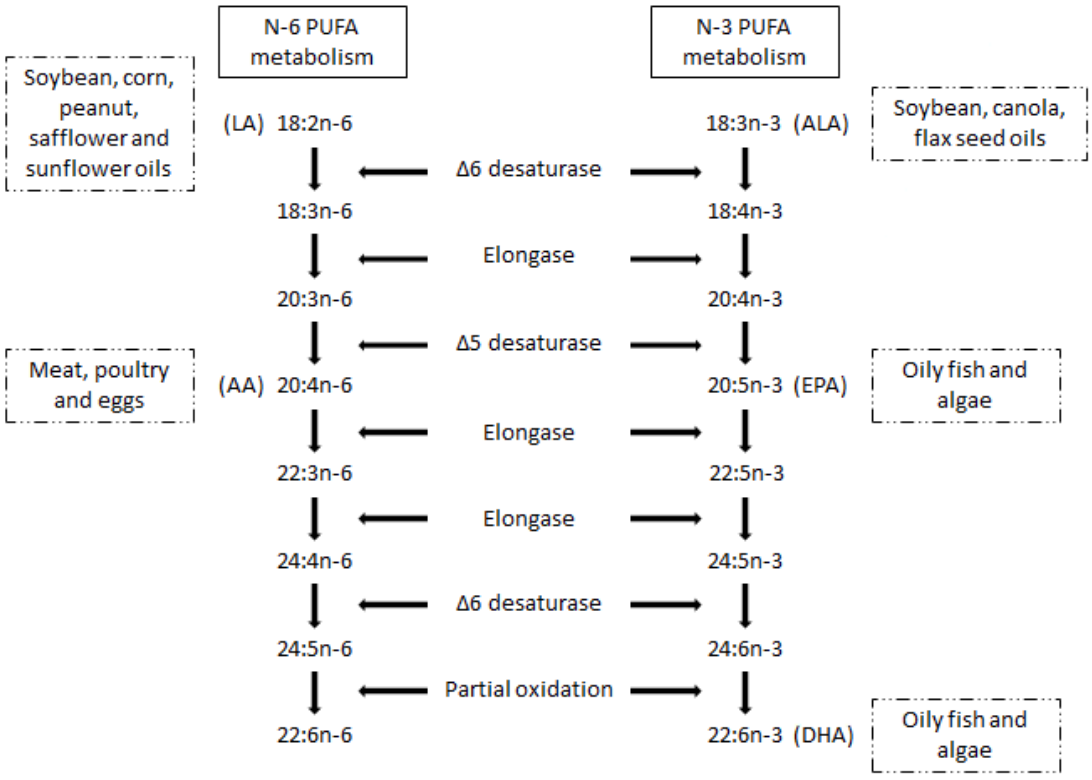


Fig 1.4. Schematic of n-6 and n-3 fatty acids desaturation and elongation. A flow chart showing how linoleic acid (LA) and ALA is metabolised by a series of desaturation (adding a double bond) and elongation (adding a 2-carbon unit) to form AA, EPA and DHA.

1.3.2.1.1 Dietary DHA

DHA and other n-3 PUFAs are found in high amounts in seafood, especially cold water oceanic oily fish. To avoid the potential mercury and other contaminations in fish, no more than 280 grams of oily fish per week is recommended according to the NHS guide for eating fish while trying to get pregnant, during pregnancy and while breastfeeding [101]. In the US, Food and Drug Administration (FDA) recommends this weekly intake of oily fish for women who might become pregnant along with pregnant and breastfeeding women is 8 to 12 ounces (approximately 227-340 grams) [102]. However, due to the potential pollutants in fish, many women reduce or stop eating fish and other seafoods during pregnancy and breastfeeding. A survey carried out in 2014 indicated that 21% of the pregnant women in the US had eaten no fish in the previous month [102]. Among the women who had fish in the previous month, 50% had fewer than 2 ounces (57 grams) weekly, and 75% reported eating fewer than 4 ounces (113 grams) a week. A supplement containing n-3 fatty acid or fish oils is an alternative way to reap the benefits of DHA and other n-3 fatty acids without risking exposure to the potential pollutants. There is no clear optimal dose of DHA and EPA supplements in NHS or FDA guidelines. Based on the oily fish intake they suggest, the range of DHA and EPA is from 200 to 2000 mg per week due to the wide range of choices of fish. Clinical trials used a wide range of doses of DHA (from 400 to 2200 mg per day) and also covered different lengths of time (from as early as 14-16 gestational weeks to delivery or up to 3 months after delivery) [103]. The main reason for starting supplementation from around 17 gestational weeks is that studies have shown that DHA accumulates in the brain of neonates during the late prenatal and early postnatal development, which corresponds to the third trimester of pregnancy in humans [104-106].

Studies on the potential neural development benefits of DHA supplementation during pregnancy and lactation to the offspring have yielded conflicting results. Some studies claim

that high DHA and eicosapentaenoic acid (EPA) have beneficial effects on child's visual and neural development [107-110]. Others hold a different view, that high DHA intake during pregnancy and lactation showed no difference in children's neurodevelopment, visual acuity or growth between placebo [111-114].

1.3.2.1.2 Mechanism of action of DHA

As mentioned previously, the mechanisms of HIE include inflammation, apoptosis, oxidative stress and toxicity. The neuroprotective mechanisms of DHA are therefore being investigated around these mechanisms. Firstly, studies have found that the anti-inflammatory effects in the brain protection against ischemic damage are provided by n-3 PUFAs especially DHA by inhibiting nuclear factor kappa B (NF-κB) pathway [115], reducing prostaglandin G/H synthase 2 (PGHS-2) and IL-1β cytokines levels [116]. Secondly, studies have also shown that DHA has an anti-apoptotic effect against brain damage [116, 117]. To investigate the anti-apoptotic effect, the protein levels of Bcl-2 and PGHS-2 have been measured by Western blotting. In addition, both *in vivo* and *in vitro* studies have demonstrated that DHA and other n-3 PUFAs can reduce ischemic neuronal injury by nuclear factor E2-related factor 2 (Nrf2) activation and heme oxygenase-1 (HO-1) up-regulation, which are associated with oxidative stress and toxicity [118].

It has been found that EPA and DHA can be metabolised and produce acute inflammation related families of molecules namely specialised pro-resolving mediators (SPMs) [119]. The families of SPMs include resolvins, protectins and maresins. A study has shown that neuroprotectin D1 (NPD1), which is produced by the oxygenation of DHA, has an important role in anti-inflammatory and neuroprotective effect against ischemic stroke [120].

1.3.2.2 Noble gases

The neuroprotective effects of noble gases, mainly xenon and argon, have been studied in recent years. Studies have shown that xenon is a potential treatment strategy of neuronal injury both *in vitro* and *in vivo* [121-123]. It is because xenon is an antagonist of NMDA and as mentioned previously, the activation of NMDA receptor plays a vital role in contributing neuronal cell death of HIE brain injury. Apart from being the antagonist of NMDA, xenon can also exert its neuroprotective effect by activating the p-Akt pathway [124] and increase the expression of Bcl-2 protein levels [125]. However, due to the high expense of xenon (around $120/100g), scientists have looked for a cheaper alternative, namely argon ($0.50/100g). It has been demonstrated in many studies that argon also has the neuroprotective effects [126-128]. The mechanisms of argon actions include it ability to act as a neuroprotective agent and it may inhibit Bcl-2, Bax, caspase-3 and NF-κB expression [129] and up-regulate extracellular signal-regulated kinases -1/2 (ERK-1/2) [130, 131]. However, ERK activation was seen *in vitro* with primary cultures of neuronal and astroglial cells and whether this activation by argon affects cellular functions like differentiation and survival in the brain *in vivo* has yet to be determined.

1.3.2.3 Stem cells therapies

Stem cells are found in all multicellular organisms; they can divide through mitosis and differentiate into diverse specialised cell types and can self-renew to produce more stem cells. The most critical features of stem cells are multipotency and self-renewal. Laboratories across the world have devoted enormous efforts into stem cell therapies to investigate their potential to repair and regenerate of damaged cells in different areas of medicine. Some studies in rodents have already shown that treatments with neural stem cells and mesenchymal stem cells after neonatal HI brain damage improved functional outcome,

reduced lesion volume, increased differentiation of recently divided cells towards neurons and oligodendrocytes and decreased proliferating inflammatory cells [132-135].

1.4 Animal models of HIE

Over the past years, scientists have used several types of animal models to mimic HIE in humans with a view to achieving two main goals: to improve our understanding of the underlying pathophysiology and the outcome of HIE damage and to create templates for testing therapeutic strategies. Vital information has been provided by animal studies with regards to mechanisms of perinatal hypoxic ischemic brain damage. To meet these two goals, the chosen animal model must have certain characteristics reflective of its target. The HIE animal model should be able to 1) mimic the aetiological condition of how the brain injury occurs, 2) show histology of injury in the developing brain and 3) show the similar short-term and long-term outcomes, which have been observed in human infants and children. Due to the fact of nature it is impossible to duplicate the exact situation that occurs in the human; however, different types of animal models are able to reproduce some features of HIE.

1.4.1 Animals models considered the development of the brain

In humans, the nervous system develops over months in utero and years after birth [19]. Many review articles and texts have compared differences in the processes of brain development between different species. A graph representing the developmental processes in the nervous system of rat, sheep and human is shown in Fig 1.5. In particular, please note the difference in duration, with timings being listed in months for the humans and sheep and only days for rats. Another table from a classic review article [4] lists the equivalent neural developmental time points of 9 species of animals, which permits translation by following a line across the columns (Table 1.4). From the table one can read that neural events occurring in a postnatal day (P) 7 rat brain is similar to a P5.6 mouse brain, a P3.1 rabbit brain and the

human limbic system at gestational day (E) 118 as well as the human posterior cortex at E155. Unfortunately, in this table there is no information about the sheep.

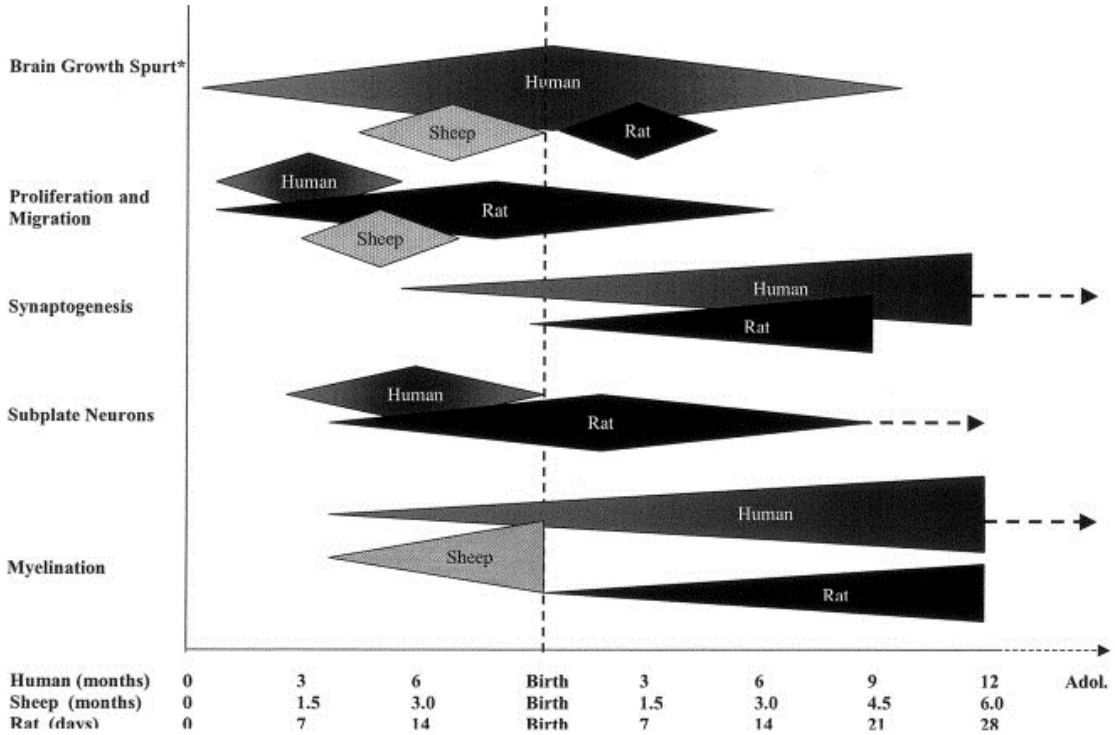


Fig 1.5. Neural developmental times of rat, sheep and human. Comparison of several parameters during brain development processes from rat, sheep and human. ∗Data are adapted from Dobbing *et al* [3].

Very few studies have used primates for the research into HIE. In 1970s, Myers *et al* established a total asphyxia and a partial ischemia models in monkeys [136]. The total asphyxia condition was created by covering their heads with a rubber sac and clamping the umbilical cord at delivery. By using this model they have found that, in order to produce any signs of neuropathologic damage, a minimal asphyxia time requirement is 12 minutes. The damages were mainly shown within the brainstem. In the partial ischemia model, monkey fetuses remained *in utero* while mothers were made hypotensive. Clinically, in this model, monkey fetuses displayed opisthotonus, decerebrate posturing as well as generalized convulsions after they have been delivered. The pathological changes at birth were

widespread cortical tissue necrosis. In the brain of the monkeys who survived, parasagittal infarction and porencephaly were observed. These observations in both models have closely mimicked the changes in human infants. However, due to the high costs and maintenance as well as the strict laboratory animal policies of using macaques, other animal models are needed.

Rodents are widely used in science laboratory. A classic HIE model has been established by Rice and Vannucci *et al* in 1981 [137]. It is one of the most robust and productive models of HIE. Based on this model, important knowledge of the condition including biochemical and pathological data in HIE research areas have been catalogued. In this model, the hypoxic condition is established by performing a unilateral common carotid artery ligation on a 7-day-postnatal rat pup. After 4-8 hours pups are then exposed to 8% oxygen at 37°C for 3.5 hours. Histologically, more than 90% of the pups suffer moderate to severe ischemic neuronal changes in the ipsilateral brain. Necrosis of white matter can also be seen in the pup brains. Furthermore, there is a 50% of chance of causing oedema in the pup brains. Due to the high efficiency of mimicking the adverse outcomes of HIE in humans, this model has been widespread and redeveloped over a number of years. Hagberg *et al* reported that there is a marked IL-1 and TNF-α response in the first 24 hours after initiating the Rice-Vannucci model [2]. As expected, a number of other research groups have also developed modifications of the Rice-Vannucci model [138-140]. With the increasing trend of using transgenic mice, the Rice-Vannucci model was adapted to the mouse by Ditelberg *et al* [141]. It has been noticed that the length of hypoxia used in neonatal mice is significantly shorter than in rats [142-145].

Table 1.4 Comparison of neural developmental times of nine different animal (including humans) species

Translating Time Across Species													
				spiny			macaque			human		
rat	hamster	mouse	rabbit	mouse	ferret	cat	limbic	other	cortex	limbic	other	cortex
(G21.5)	(G15.5)	(G18.5)	(G31)	(G39)	(G41)	(G65)	(G165)			(G270)		
9.0	8.1	8.3	9.9	10.4	13.9	14.7	19	20	23	23	25	29
9.5	8.5	8.7	10.5	11.1	15.0	16.0	21	22	26	26	27	32
10.0	8.9	9.1	11.1	11.8	16.2	17.3	23	24	28	28	30	35
10.5	9.3	9.5	11.7	12.5	17.4	18.6	24	26	31	30	33	39
11.0	9.7	9.9	12.4	13.2	18.6	19.8	26	28	33	33	35	42
11.5	10.1	10.3	13.0	13.8	19.7	21.1	28	30	36	35	38	45
12.0	10.4	10.7	13.6	14.5	20.9	22.4	30	32	38	38	41	48
12.5	10.8	11.1	14.2	15.2	22.1	23.7	32	34	41	40	43	52
13.0	11.2	11.5	14.9	15.9	23.2	25.0	34	36	43	43	46	55
13.5	11.6	11.9	15.5	16.6	24.4	26.3	35	38	45	45	48	58
14.0	12.0	12.3	16.1	17.3	25.6	27.6	37	40	48	47	51	61
14.5	12.4	12.7	16.7	18.0	26.8	28.8	39	42	50	50	54	65
15.0	12.7	13.2	17.3	18.7	27.9	30.1	41	44	53	52	56	68
15.5	13.1	13.6	18.0	19.4	29.1	31.4	43	46	55	55	59	71
16.0	13.5	14.0	18.6	20.1	30.3	32.7	45	48	58	57	62	74
16.5	13.9	14.4	19.2	20.8	31.4	34.0	47	50	60	60	64	78
17.0	14.3	14.8	19.8	21.5	32.6	35.3	48	52	63	62	67	81
17.5	14.6	15.2	20.5	22.1	33.8	36.5	50	54	65	64	70	84
18.0	15.0	15.6	21.1	22.8	34.9	37.8	52	56	68	67	72	87
18.5	15.4	16.0	21.7	23.5	36.1	39.1	54	58	70	69	75	91
19.0	BIRTH	16.4	22.3	24.2	37.3	40.4	56	60	73	72	78	94
19.5	P0.7	16.8	22.9	24.9	38.5	41.7	58	62	75	74	80	97
20.0	P1.1	17.2	23.6	25.6	39.6	43.0	60	64	78	77	83	100
20.5	P1.4	17.6	24.2	26.3	BIRTH	44.3	61	66	80	79	86	104
21.0	P1.8	18.0	24.8	27.0	P1	45.5	63	68	82	82	88	107
BIRTH	P2.2	BIRTH	25.4	27.7	P2.1	46.8	65	70	85	84	91	110
P1	P3	P0.7	26.7	29.1	P4.5	49.4	69	74	90	89	96	117
P2	P3.7	P1.5	27.9	30.4	P6.8	52.0	72	78	95	94	101	123
P3	P4.5	P2.3	29.2	31.8	P9.2	54.5	76	82	100	99	107	130
P4	P5.3	P3.1	BIRTH	33.2	P11.5	57.1	80	86	105	103	112	136
P5	P6	P3.9	P0.6	34.6	P13.9	59.7	84	90	110	108	117	143
P6	P6.8	P4.8	P1.9	36.0	P16.2	62.2	87	94	114	113	123	149
P7	P7.6	P5.6	P3.1	37.4	P18.5	BIRTH	91	98	119	118	128	155
P8	P8.3	P6.4	P4.4	BIRTH	P20.9	P2.4	95	102	124	123	133	162
P9	P9.1	P7.2	P5.6	P1.1	P23.2	P5	98	107	129	128	139	168
P10	P9.9	P8	P6.9	P2.5	P25.6	P7.5	102	111	134	133	144	175
P11	P10.6	P8.8	P8.1	P3.9	P27.9	P10.1	106	115	139	138	149	181
P12	P11.4	P9.6	P9.4	P5.3	P30.3	P12.7	109	119	144	142	154	188
P13	P12.2	P10.4	P10.6	P6.6	P32.6	P15.2	113	123	149	147	160	194
P14	P12.9	P11.2	P11.8	P8	P34.9	P17.8	117	127	154	152	165	201
							122	133	161	159	173	211
							126	137	BIRTH	164	178	217
										169	184	224
										174	189	230
										179	194	237
										184	199	243
										189	205	250
										194	210	256
										198	215	263
										203	221	BIRTH

Dates in bold typeface are gestational days; dates in regular typeface are listed as postnatal days. Total gestation times for each species are listed at the top of each column with a 'G'. . ∗Data are adapted from Clancy *et al* [4].

1.4.2 Animals models considered the intrauterine environment

Although the Rice-Vannucci model and its various modified versions have ticked many boxes of the goals for a successful animal model of HIE, the fact that it has been built on a P7 animal instead of on a fetus remaining *in utero* could have unforeseen results. For this reason, scientists have established a true intrauterine HI models in the fetal sheep [146-148]. However, only some of the fetal sheep showed clinical evidence of brain injury. This might due to the fact that the brain of a near-term fetal sheep (> E125) is relatively mature and more closely resembles that of a newborn lamb (145 gestational days) [149, 150].

As an alternative, Derrick *et al* has developed a global HI model in the near-term rabbit that nicely mimics acute placental insufficiency in humans [151]. New-born pups in the hypoxic situation show significant impairment in motor ability and histology. Nevertheless, as the rat is the most commonly used laboratory animal, scientists have also modified this model in the rat [152-155]. The present study is the first to try and establish an in utero model of HIE in the mouse.

1.5 Aims

1) To establish a clinically relevant model of perinatal HIE in mice and use this model to study ways of alleviating this harmful clinical condition.

2) To define whether LPS induced maternal systemic inflammation can worsen the outcome of neonates who had previously suffered HIE brain damage.

3) To determine if and how a DHA enriched maternal diet improves the outcome of neonates who suffered HIE brain damage.

1.6 Hypotheses

1) An intrauterine mouse model can be used to mimic the histological and clinical outcomes of HIE in humans.

2) Systemic inflammation in the latter stages of pregnancy results in a worse outcome for pups exposed to HIE.

3) Giving a DHA enriched diet to dams could improve both short-term and long-term outcomes of neonates who suffered HIE brain damage.

Chapter 2. Materials and Methods

2.1 Animals

All animal studies were approved by the Ethics Committee of Imperial College London and University College London and performed under the UK Home Office Licences 70/6966 or 70/7518. All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986, United Kingdom and carried out in compliance with the ARRIVE guidelines. Animals were housed in individually ventilated cages or open cages at 21 ± 1°C, on a 12:12 hours light/dark cycle, and were administered food and water *ad libitum*.

2.1.1 Time-mated Mice

CD1 wild type outbred time-mated mice were obtained from Charles River Laboratories (CR, Wilmington, Massachusetts, USA) at different gestational ages (see Table 2.1 below) and left to acclimatise for a minimum of 7 days before undergoing any regulated procedure.

Table 2.1 Different gestational ages of mice acquired from CR for different studies

Studies	Gestational age (E) of mice
Development of HIE model	E8 - E11
DHA Maternal supplementation	E0 - E1
LPS induced systemic inflammation	E6 – E7

2.2 Animal Model

2.2.1 HIE model

A model of HIE in rats was originally developed by Bjelke *et al* in 1991 [152]. On the last day of gestation (E18 for CD1 mouse), pregnant mice were sacrificed by cervical dislocation. The pups from one uterine horn were delivered immediately by caesarean section (CS) and were used as the CS control group. The second uterine horn was clamped, including the fetus and vessels, then cut and placed in a 0.9% sodium chloride (FKE1323, Baxter International, Deerfield, Illinois, US) water bath at 37°C for 10 to 16 minutes, after which, pups were rapidly delivered. The time of asphyxia was measured from the time when the uterus was clamped, and the uterus including the fetuses, was placed into the water bath for a specified period of time until it was taken out. All pups with or without hypoxia were transferred and placed in an incubator at 37°C for 1 hour whilst they received manual stimulation to maintain spontaneous respiration. The newborn pups were transferred to surrogate mothers who had previously given birth naturally. Some pups, from the surrogate mothers, which were born one day in advanced were used as naïve controls. This process is shown diagrammatically in Fig 2.1.

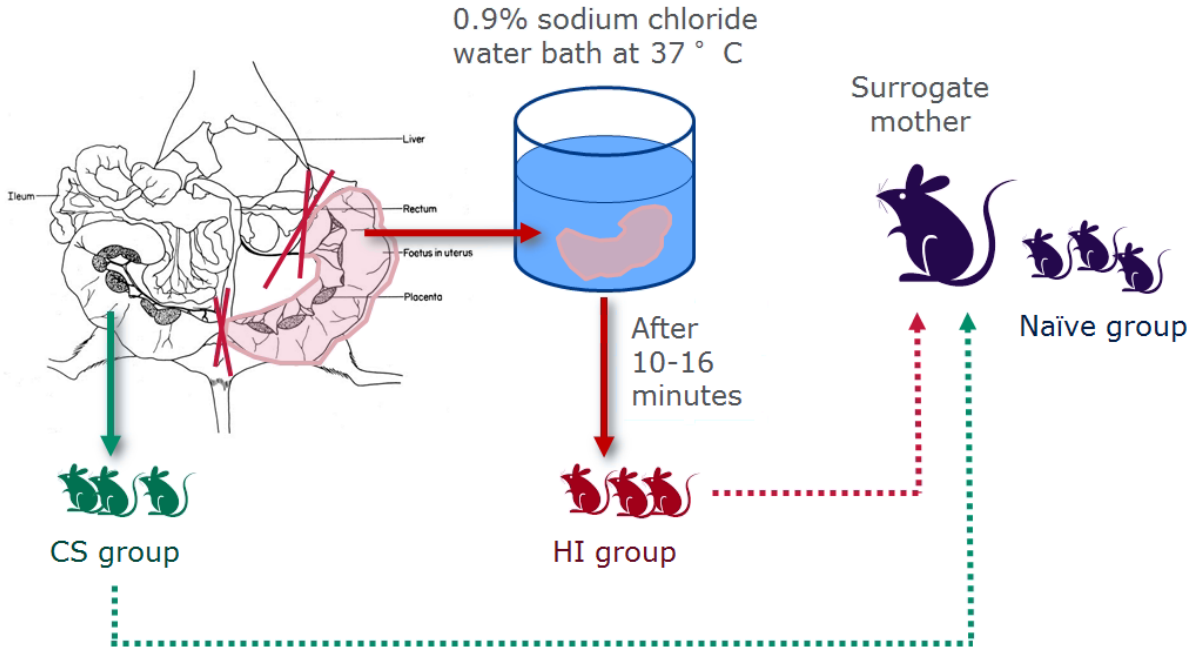


Figure 2.1. The procedure to establish the HIE mouse model.

2.2.2 DHA maternal supplementation

All diets were made by Special Diets Services (Essex, UK). Mice were fed with a maintenance diet (VRF1, 801900) while in the care of Charles River and Rat and Mouse No.1 maintenance autoclavable diet (RM1, 801151) after they were transferred to the animal facility.

On gestational day 2 (E2) mice were randomly assigned into two experimental groups: a control group which were fed a control diet or a DHA enriched group which were fed on the control diet supplemented with 1.5% DHA as measured in relation to the total fat content of the diet. Both experimental diets were specially made according to Irina Fedorova *et al* [156]. The HIE model was used as described previously in section 2.2.1. The same diets were fed to the dams and pups after birth until postnatal day 21 when pups were weaned.

2.2.3 LPS induced systemic inflammation

Pregnant dams were randomly divided into two LPS groups with 100µg/kg or 200µg/kg of Escherichia coli (0127:B8, Sigma-Aldrich, St. Louis, Missouri, USA) intravenous (i.v.) injected in a total volume of 100µl sterile saline (sodium chloride 0.9%, FKE1323, Baxter, Deerfield, Illinois, United States) and 2 control groups: a group with no injections and a group injected with 100 µl i.v. sterile saline. The pregnant mice were i.v. injected without being anaesthetised. The i.v. injections were performed once daily from E14 to E17.

2.3 Mouse Tissue Collection and Processing

Mice pups were culled via decapitation at 1, 2, 6, 12, 24, 72 and 168 hours after birth while adult mice were culled via terminal anaesthesia. Pup brains were taken and stored at -80°C or at 4°C in 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, Missouri, USA). For plasma and red blood cell collection, adult mice were culled via terminal anaesthesia then exsanguination by cardiac puncture. 100 µl of 0.25M ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, Missouri, USA) was added into 1 mL of whole blood and then was centrifuged immediately at 1000x g for 10 minutes to obtain plasma and red blood cell (RBC), then snap frozen on dry ice and stored at -80°C. For adult brain collection, mice were under terminal anaesthesia then perfused with phosphate-buffered saline (PBS) (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) followed by 4% PFA via vascular system. Perfused brains stored at 4°C in 4% PFA for at least 24 hours for post fixation before use.

2.4 Quantitative Polymerase Chain Reaction (qPCR)

Brain tissue was homogenised and total RNA was extracted with a RNA purification kit (RNeasy Kit, Qiagen, Venlo, Netherlands) according to the manufacturer's guidelines. After

quantification by NanoDrop, 1.0μg RNA was reverse transcribed with oligo dT random primers using MuLV reverse transcriptase (Life Technologies, Carlsbad, California, USA). Primer sets were designed using primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and purchased from Invitrogen Corporation (Carlsbad, California, USA). Primers used in this study are listed in Table 2.2. 2 μL of diluted cDNA was amplified with Power SYBR® Green PCR Master mix (Life Technologies, Carlsbad, California, USA) in a final volume of 20 μL. The real-time PCR was performed on a Rotor-Gene Q system (Qiagen, Hilden, Germany) for 50 - 55 cycles: 20 s at 95°C, 20 s at 60°C and 20 s at 72°C. Quantification cycle values were extrapolated with the Rotor-Gene Q series software (Qiagen, Hilden, Germany) using standards made from the individual primer pairs.

Table 2.2 Primer sequences used for qPCR

Primers	Forward (F) and Reverse (R) sequences (5'-3')	Genebank/EMBL Accession no.	Start - end and product size (bp)
IL-1β	F: 5'GACCTTCCAGGATGAGGACA3' R:5' AGCTCATATGGGTCCGACAG3'	NM_008361	314 - 496 = 183
IL-6	F: 5'AGTTGCCTTCTTGGGACTGA3' R: 5'TCCACGATTTCCCAGAGAAC3'	DQ788722.1	33 - 191 = 159
IL-10	F: 5'CCAAGCCTTATCGGAAATGA3' R: 5'GCTCCACTGCCTTGCTCTTA3'	NM_010548.2	307 - 488 = 181
IL-2	F: 5'GCAGGCCACAGAATTGAAAG3' R: 5'TCCACCACAGTTGCTGACTC3'	NM_008366.3	294 - 500 = 207
IL-4	F: 5'CCTCACAGCAACGAAGAACA3' R: 5'TGGACTCATTCATGGTGCAG3'	M25892.1	252 - 445 = 194
CXCL-1	F: 5'GCCTATCGCCAATGAGCTG3' R: 5'AAGGGAGCTTCAGGGTCAAG3'	NM_008176	71 - 321 = 251
CXCL-2	F: 5'AGTGAACTGCGCTGTCAATG3' R:5' TTCAGGGTCAAGGCAAACTT3'	BC119511.1	114 - 266 = 153
TNF-α	F: 5'CAAACCACCAAGTGGAGGAG3' R: 5'GTGGGTGAGGAGCACGTAGT3'	NM_013693	446 - 624 = 179
PGHS-2	F: 5'TGCAGAATTGAAAGCCCTCT3' R: 5'GCTCGGCTTCCAGTATTGAG3'	NM_011198.4	1615 - 1795 = 181
GAPDH	F: 5'ACTCCACTCACGGCAAATTC3' R: 5'TCTCCATGGTGGTGAAGACA3'	NM_001001303	201 - 371 = 171
β-actin	F: 5'AGGCTGTGCTGTCCCTGTAT3' R: 5'GCTGTGGTGGTGAAGCTGTA3'	NM_007393	490 - 691 = 202

2.5 Protein Analysis

2.5.1 Western blotting

Pup brain samples were mechanically homogenized in cell lysis buffer (#9803, Cell signaling, Danvers, Massachusetts, USA). The cell lysates were centrifuged, the supernatant collected and total protein concentration was quantified using the DC protein assay (Bio-Rad Laboratories, Inc., Hercules, California, USA). The protein extracts (40µg/sample) were heated, denatured and loaded onto a polyacrylamide 4 to 15% precast gel (#4561086, Mini-PROTEAN, Bio-Rad Laboratories, Inc., Hercules, California, USA) for electrophoresis and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc., Hercules, California, USA) The membrane was treated with blocking solution (5% dry milk (Marvel, Premier Foods Group Ltd, London, UK) in tris-buffered saline with Tween® 20 (TBST) (Sigma-Aldrich, St. Louis, Missouri, USA) for 1 hour and probed with the primary antibodies in TBST overnight at 4°C, followed by HRP-conjugated secondary antibodies for 2 hours. The blots were visualized with enhanced chemiluminescence (ECL) system (Santa Cruz, Dallas, USA) and analysed with GeneSnap (Syngene, Cambridge, UK). Protein band intensity was nomalised with GAPDH and expressed as ratio of control for data analysis.

Table 2.3 Antibodies used for Western blotting

Antibodies	Product code	Working dilution	Supplier
Cleaved Caspase-3 (Asp175)	#9664	1:500	Cell Signaling, Danvers, Massachusetts, USA
Bcl-2	#3869	1:1000	Cell Signaling, Danvers, Massachusetts, USA
Bax	#2772	1:1000	Cell Signaling, Danvers, Massachusetts, USA
Phospho-NF-κB p65 (Ser536)	#3033	1:500	Cell Signaling, Danvers, Massachusetts, USA

Phospho-NF-κB p65 (Ser468)	#3039	1:500	Cell Signaling, Danvers, Massachusetts, USA
Acetyl-NF-κB p65 (Lys310)	#3045	1:500	Cell Signaling, Danvers, Massachusetts, USA
NF-κB p65 (D14E12) XP	#8242	1:1000	Cell Signaling, Danvers, Massachusetts, USA
HIF-1α	NB100-449	1:2000	Novus Biologicals, Littleton, Colorado, USA
GAPDH	#2118	1:10000	Cell Signaling, Danvers, Massachusetts, USA
Anti-rabbit IgG, HRP-linked Antibody	#7074	1:2000	Cell Signaling, Danvers, Massachusetts, USA

2.5.2 Multiplex Analysis

2.5.2.1 Chemokine and cytokine detection

Tissue lysis buffer was prepared using a Bio-Plex Cell Lysis Kit (#171-304011, Bio-Rad Laboratories, Inc., Hercules, California, USA) Mouse tissues (mouse maternal tissue samples including those from the lung, liver, uterus, placenta and pup brains) were then homogenised via 4 periods of 20 seconds at 5000rpm at 40°C using a Precellys®24 (Stretton Scientific Ltd., Derbyshire, UK) homogeniser with 5 minutes intervals. Protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, California, USA). 500µg of protein samples were diluted 1:1 with Bio-Plex sample diluents and were assayed in each well. Selected analytes, including IL-1α, IL-1β, IL-6, IL-4, IL-10, IL-12 p40, IL-12 p70, granulocyte-colony stimulating factor (G-CSF), interferon gamma (IFN-γ), CXCL-1, CXCL-2, CCL-2, CCL-5 and TNF-α, were selected from the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (M60009RDPD, Bio-Rad Laboratories, Inc., Hercules, California, USA). Multiplex assays were performed according to the manufacturer's instructions (#10014905, Bio-Rad Laboratories, Inc., Hercules, California, USA). Assays were read using a Bio-Plex© MAGPIX© reader.

2.5.2.2 Apoptosis related proteins and HIF-1α detection

Tissue lysis buffer was prepared by adding protease inhibitors (#535140, EMD Millipore, Billerica, Massachusetts, USA) into MILLIPLEX MAP Lysis Buffer (#43-040, EMD Millipore, Billerica, Massachusetts, USA). Mouse pup brain tissues were then homogenised via 4 periods of 20 seconds at 5000rpm at 4°C using a Precellys®24 (Stretton Scientific Ltd., Derbyshire, UK) homogeniser with 5 minutes intervals. Protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, California, USA). For apoptosis related proteins detection, 25µg of protein diluted 1:1 with

MILLIPLEX Assay buffer 2 was assayed per well. A mouse Multiplexing MILLIPLEX assay, including phospho-p38, phospho-bad, phospho-STAT3 and cleaved caspase-3, was performed according to the manufacturer's instructions (#48-602MAG, EMD Millipore, Billerica, Massachusetts, USA). For HIF-1α detection, 10μg of protein diluted 1:2 with MILLIPLEX Assay buffer 1 was assayed per well and performed according to the manufacturer's instructions (#HGPMAG-27K, EMD Millipore, Billerica, Massachusetts, USA). Assays were read using a Bio-Plex© MAGPIX© reader.

Table 2.4 Analytes using in MILLIPLEX assay

Analytes	**Product code**
Phospho-p38	46-610MAG
Phospho-bad	46-694MAG
Phospho-STAT3	46-624MAG
Cleaved caspase-3	46-6-4MAG
β-tublin	46-713MAG
HIF-1α	HGPMAG-27K

2.6 Immunohistochemistry, TUNEL staining and Histology

All the photomicrographs in this study were taken using an Olympus BX-60 microscope and captured with a Zeiss KS-300 colour 3CCD camera. Immunofluorescence was quantified using Image J (National Institutes of Health, Maryland, USA).

2.6.1 Immunohistochemistry

Frozen sections (10µm) were incubated in a blocking solution of 10% normal donkey serum in 0.1M phosphate -buffered saline with Tween® 20 (PBST) (Sigma-Aldrich, St. Louis, Missouri, USA) then incubated overnight with primary antibodies in PBST. Immunoreactivity was detected using fluorescein conjugated secondary antibodies for 1 hour. Slides were counterstained and mounted with Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories Ltd, Peterborough, Cambridgeshire, UK).

Table 2.5 Antibodies used for immunohistochemistry

Antibodies	Product code	Working dilution	Supplier
Cleaved Caspase-3 (Asp175)	#9664	1:200	Cell Signaling, Danvers, Massachusetts, USA
Glial fibrillary acidic protein (GFAP)	ab7260	1:500	Abcam, Milton, Cambridge, UK
Donky anti-rabbit IgG (FITC)	AP182F	1:200	EMD Millipore, Billerica, Massachusetts, USA
Donky anti-rabbit IgG (Rhodamine)	AP182R	1:200	EMD Millipore, Billerica, Massachusetts, USA

2.6.2 Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) staining

TUNEL staining (paraffin sections, 8µm) was performed using a commercially available kit (ApopTag® Plus Peroxidase *in situ* Apoptosis Detection Kit, S7101, EMD Millipore, Billerica, Massachusetts, USA) according to the manufacturer's instructions. Briefly, sections

were pre-treated with proteinase K (Sigma-Aldrich, St. Louis, Missouri, USA) for 15 minutes then quenched in 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, Missouri, USA) for 5 minutes. After 1 minute incubation in equilibration buffer, sections were incubated in working strength TdT enzyme for 1 hour at 37°C. The reaction was terminated by stop/wash buffer then anti-digoxigenin conjugate was then applied for 30 minutes. Colour was developed by 3, 3 -diaminobenzidine (DAB) (SK-4100, Vector Laboratories Ltd, Peterborough, Cambridgeshire, UK) followed by counterstaining with methyl green (Vector Laboratories Ltd, Peterborough, Cambridgeshire, UK) according to the manufacturer's guidelines.

2.6.3 Haematoxylin and Eosin (H&E) Staining

Paraffin embedded sections (8µm) or frozen sections (15µm) were stained with haematoxylin and eosin (H&E). Briefly, paraffin sections were deparaffinised then re-hydrated with different grades of ethanol (VWR, Radnor, Pennsylvania, USA) then washed in distilled water while frozen sections were washed with distilled water. Slides were then stained in Harris haematoxylin solution (Sigma-Aldrich, St. Louis, Missouri, USA) for 8 minutes followed by washing in running tap water for 5 minutes. After being differentiated in 1% acid alcohol for 30 seconds and a 1-minute wash with running tap water, slides were placed in 0.2% ammonia water for 30 seconds to 1 minute for blueing. After washing and dehydration slides were counterstained in 1% eosin solution (Raymond A Lamb Ltd., East Sussex, UK) for 30 seconds to 1 minute. Slides were dehydrated through 95% alcohol and 2 changes of absolute ethanol before clearing in 2 changes of xylene (Fisher Scientific, Waltham, Massachusetts, USA). Slides were mounted with xylene based mounting medium (DPX mounting medium, Thermo Scientific, Waltham, Massachusetts, USA). Cell counting was performed by an investigator who was blinded to the interventional groups.

2.7 Fatty Acid Analysis

The total lipids of from samples of maternal blood, pup brain and the diets used were extracted by the method of Folch *et al* [157]. Briefly, samples were homogenised in chloroform/ methanol (Fisher Scientific, Waltham, Massachusetts, USA), 2:1 v/v, + 0.01% butylated hydroxyl toluene (BHT) (Fisher Scientific, Waltham, Massachusetts, USA) and then stored at 4°C for 24 hours. After filtering, 25% v/v of 0.85% saline (Fisher Scientific, Waltham, Massachusetts, USA) was added and stored overnight at 4°C which allowed partitioning into a lower solvent phase which was then transferred into a round bottomed flask and evaporated under a stream of nitrogen. Fatty acid methyl esters (FAMEs) were prepared from the lipid fraction by reacting with acetyl chloride–methanol solution in a sealed vial at 70°C for 3 hours. These esters were separated and analysed by gas-liquid chromatography (Agilent Technologies, Santa Clara, California, USA) with hydrogen as carrier gas. FAMEs were identified by comparison of retention times with authentic standards. The value of the individual essential fatty acid was expressed as a percentage of total FAMEs.

2.8 Long-term Neurocognitive Outcome

In all the behavioural tests used in this study, mice were transported to the test room in their home cages 30 minutes before the first trial started for acclimatisation.

2.8.1 Morris Water Maze

Spatial memory was evaluated daily (starting on post-natal day 45, P45) for 6 continuous days using a Morris water maze (WM) [158]. Each individual mouse performance was recorded by a computerized video tracking system (EthoVision®; Noldus, Netherlands). Briefly, WM consisted of a circular pool 110 cm in diameter and 60 cm in height, filled to a

depth of 30 cm with water (24±0.5°C). A hidden platform (1 cm beneath the water surface) was placed in the centre of one quadrant. Mice were placed in the water in a dimly lit room with distinctive 2D and 3D visual cues placed on the walls of the room. Four trials were performed for each mouse every day. Training was repeated for 5 consecutive days. The time (latency, cut-off time 60 seconds) taken for the mice to locate the hidden platform was recorded. On assessment day (day 6), each animal was subjected to a probe trial (60 seconds cut-off), where they were tested in the absence of the platform. The time spent in the quadrant where the platform was located during the previous trials was recorded. The path length and swim speed during 6 days were also recorded by the tracking system.

2.8.2 T-maze continuous alternation task

The T-maze consisted of a start arm (40 × 10 × 15 cm) and two identical goal arms (30 cm × 10 cm × 15 cm). Arms could be blocked by 3 guillotine-type doors 1 cm from the joint point of the T maze. The test session consisted of one forced trial and 15 subsequent free-choice trials. In the first trial, doors of the goal arms were lifted and the door at the start arm was closed. The mouse was placed at the end of the start arm and after 5 s of confinement, the door was lifted and the mouse was allowed to explore the T-maze. As soon as the mouse entered one goal arm (including all four feet and the tip of the tail), the other goal arm was closed. The next free choice trail was started when the mouse returned to the start point and the door at the goal arm was lifted straight away. The T-maze was cleaned with water before each mouse started its session. The consecutive choices made by the mice were recorded. The overall alternation rate was calculated as a percentage (number of alternations/total number of choices). Each individual mouse performance was recorded by a computerized video tracking system (EthoVision®, Noldus, Netherlands).

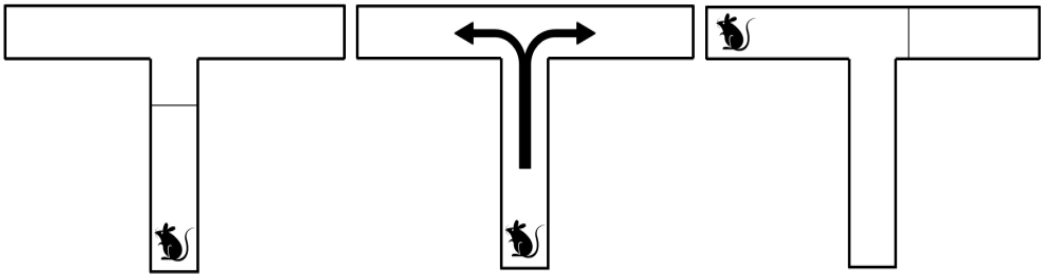


Figure 2.2. T-maze alternation tasks.

2.8.3 Fear Conditioning

Fear conditioning was conducted in two identical conditioning chambers (17.78 cm × 19.05 cm × 38.10 cm) housed in a sound attenuating box (Med Associates Inc, St Albans, VT) with a video camera to record animal responses. The foot shock (unconditional stimuli, 0.70 mA) was delivered through a stainless steel grid floor. On P55, the training sections were performed. Mice were acclimatized for 120 seconds before exposure to six trials of paired conditional stimuli (a high pitched tone, for 40 seconds at 90 dB) and unconditional stimuli (2 seconds). The trace interval was 20 seconds between tone and shock, with pseudorandom inter-training intervals (iti) of between 30 and 45 seconds. The total training time for each animal was 772 seconds (Fig.2.3). 3 days after the training day, the hippocampal dependent and hippocampal independent memories were assessed. Assessment of contextual memory to test hippocampal dependence memory was performed in the same context (no roof, no floor, light off, fan on, scent of mint (Sainsbury, Holborn, London, UK) for 2 minutes without both conditional and unconditional stimuli while hippocampal independent memory was assessed in a novel context (with roof and floor, light on, fan off, scent of vanilla (Sainsbury)) with the conditional stimuli. The animals had a one-hour interval between the assessments of

contextual memory and hippocampal independent memory. Freezing was defined as the absence of any visible movement, except that required for respiration, recorded by Video Freeze Version 2.1.0 (Med Associates Inc., St Albans, VT). The percentage of freezing-time, an indicator of memory formation, was assessed by an independent operator for further statistical analysis using the video recordings and tabulated data generated by the software.

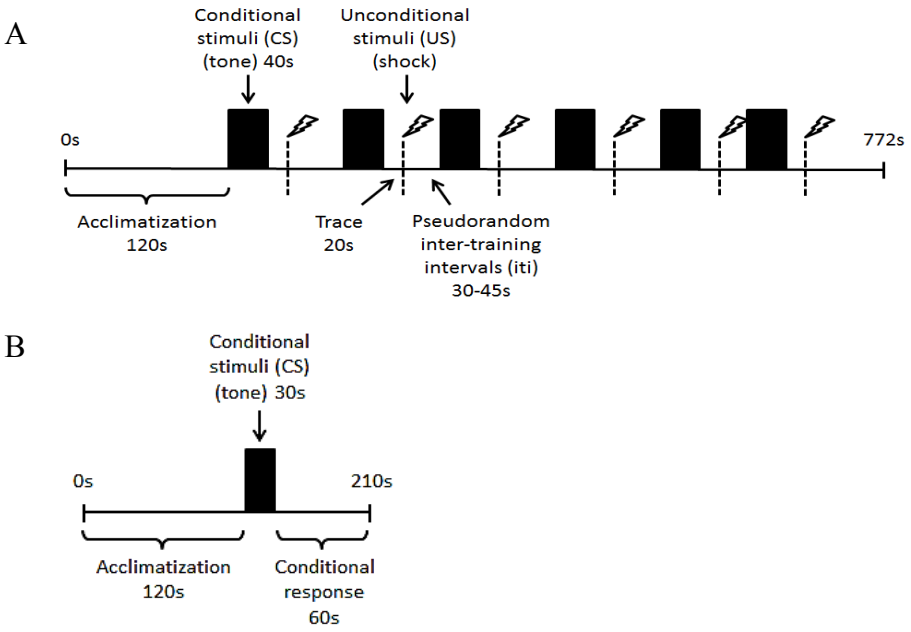


Figure 2.3. Time course for the training section of the fear conditioning test. A. Time course of training section of fear conditioning test. **B**. Time course of the assessment of hippocampal independent memory in the fear conditioning test.

2.8.4 Open Field Test

The protocol of open field test was first developed by Calvin S. Hall [159]. A square non-transparent open field apparatus (30 cm x 30 cm x 30 cm) was used for these tests. Geometric grids were marked beneath the bottom of the apparatus to define the centre zone (Fig 2.4). Each mouse was placed in the periphery of the apparatus and allowed to explore the box freely for 10 minutes under indirect dim light. The box was cleaned with water before each mouse started its session to remove all traces of odour from the previous test animal. A

remote camera (Sony, Minato, Tokyo, Japan) was positioned over the top of the box and video-tracking software (EthoVision®, Noldus, Netherlands) was used. Total distance, average speed and time spent in different zones of the field were recorded.

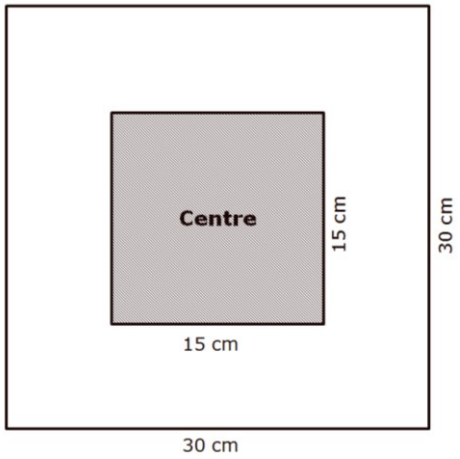


Figure 2.4. Open field test. The experimental design for the open field box.

2.8.5 Novel Object Recognition (NOR) task

The NOR task was conducted in an open field arena with two different kinds of objects [160]. Both objects were generally consistent in height and volume, but were different in shape and appearance (yellow ping pong ball and blue shot glasses, Amazon, Seattle, Washington, UK). Mice aged P90 were studied. The test had two phases, a sample trial and a test trial (Fig 2.5). All objects were made of plastic and disposable, and the field thoroughly cleaned in between animals to avoid odour cues. The test trial was given 4 hours later where the mouse was presented with a familiar object (seen in the sample trial) and a novel object (different in shape and colour). In both trials, an exploration was defined as the nose of the animal directed 2 cm or closer to the object and/or touching the object. Video-tracking software (EthoVision®, Noldus, Netherlands) was used to record the trials to avoid bias.

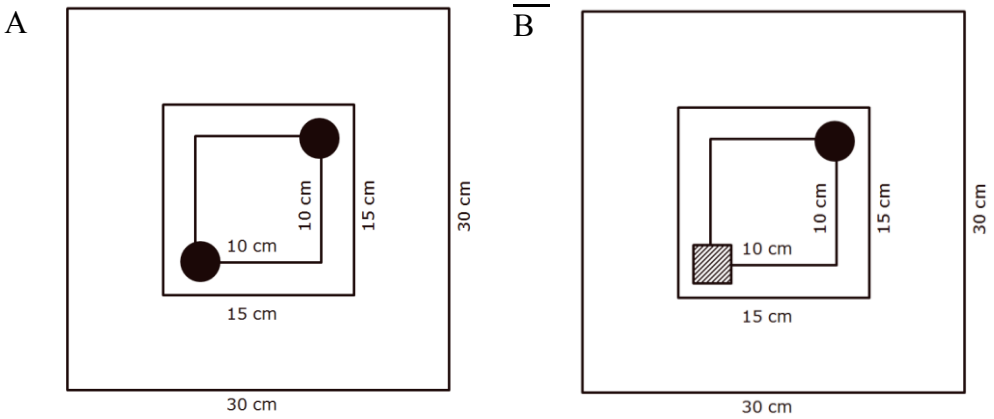


Figure 2.5. NOR task. A. The experiment design for sample trial. B. The experiment design for test trial.

2.9 Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., California). The results are expressed as mean ± SEM. Survival rates were analysed by Chi-square. The distribution of data was determined using Kolmogorov-Smirnov test. Normally distributed data were analysed using parametric tests: an unpaired t test for two groups and a one way analysis of variance (one way ANOVA) followed by a Bonferroni *post hoc* test for three groups or more. Data that were not normality distributed were analysed using a Mann Whitney test for unpaired data and when comparing three or more groups a Friedman's Test, with a Dunn's Multiple Comparisons *post hoc* test. The significance level was set at $p < 0.05$.

Chapter 3. The Hypoxic ischaemic encephalopathy mouse model

3.1 Introduction

Hypoxic ischaemic encephalopathy (HIE) is a major cause of acute mortality and chronic neurological morbidity in newborns. HIE may lead to different brain disorders, including spasticity, epilepsy, attention deficit disorders and may form psychiatric and neurodegenerative diseases [28, 161, 162].

Over the past years, scientists have used several types of animal studies to try to understand the pathophysiology of HIE. Vital information has been provided by animal studies with regards to mechanisms of perinatal hypoxic ischemic brain damage. The most commonly used animal model of perinatal asphyxia is a rat model introduced by Vannucci's group in the early 1980s. In that model, the brain damage is created on a 7-day postnatal (P7) rat pup by performing the unilateral common carotid artery ligation with subsequent exposure to 8% oxygen. The Vannucci rat model has proved useful for numerous studies of perinatal hypoxic ischemic brain damage and presently is utilized by many investigators. The 7-day-old rat was originally chosen because, at this stage of rat brain development, the rat's brain is histologically similar to that of a 32 to 34 weeks gestation human fetus or newborn infant. However, one of the greatest limitations of this model is that it did not mimic a true intrauterine HI environment. An intrauterine HIE model in rat was originally developed by Bjelke *et al* in 1991 [152]. In this model, on the last day of gestation, rats undergo a hysterectomy. In order to produce asphyxia, the entire uterus including the fetuses is placed in a 37°C water bath for 3 time intervals (14-15 minutes, 15-16 minutes and 16-17 minutes). After the time has elapsed, the uterus is taken out and opened to deliver the pups. Pups are then kept on a heat pad for 15-20 minutes to recover before being placed with a surrogate mother. Two control groups are used for comparison: a naïve group of animals which are born spontaneously by the surrogate mother and another group of animals where Caesarean section (CS) is performed under normoxic condition and then placed with the surrogate

mothers. In the current study, a similar method was used to induce perinatal asphyxia in the term mouse. The mechanisms of HI brain damage are heterogeneous. A complex series of pathological events including apoptosis, inflammation, oxidative stress and excitotoxicity are known to precipitate neuronal demise and subsequent neurological dysfunction [138, 163-165]. The mechanism of neuronal cell death after hypoxic ischemic injury involves necrosis and apoptosis. Cerebral inflammation, characterized by activation of microglia and macrophages, leukocyte infiltration, release of pro-inflammatory cytokines and increased expression of endothelial adhesion molecules [166, 167] can also make a substantial contribution to hypoxia-induced brain injury [138]. Hypoxia occurs immediately upon cerebrovascular occlusion and contributes to the progression of ischemic cascade. Hypoxia-inducible factor 1-alpha (HIF-1α) is a protein whose expression in tissue, reflects an environment with insufficient oxygen. Perinatal HIE results in acute hypoxia-induced neuronal death in the hippocampus area [152, 153, 168]. The neuronal death is followed by a delayed but prolonged apoptotic-like neuronal death in the surrounding zone [163, 164]. Recent data suggest that apoptosis may play a more important role in the neonatal brain than necrosis after injury [169].

The major concern in human HIE is the long term effects on the brain. Several studies have addressed the behavioural disturbances induced in rats subjected to acute perinatal asphyxia, mainly addressing motor function, emotional behaviour and spatial memory using Morris water maze, T maze, open field test (OFT) and fear conditioning test [154, 170-173]. Some other studies have used novel object recognition (NOR) tests and other behavioural tests that have no stressful cues or primary reinforcers, such as swimming, food deprivation or electric shocks, to measure the non-spatial memory of rodents who suffered HI brain damage [174-176].

3.2 Aim and Hypothesis

To establish a clinically relevant model of perinatal HIE in mice and use this model to study ways of alleviating this harmful clinical condition.

3.3 Materials and Methods

The HIE model was established as previously described in Chapter 2 (2.2.1). Clinically, the Apgar score is a standard method to summarise the health of a newborn infant [177, 178]. The score evaluates a newborn infant based on 5 criteria: appearance (skin colour), pulse (heart rate), grimace (reflex irritability), activity and respiration. A score of 7 or higher indicates that the baby's condition is good to excellent while a very low Apgar score (<3) is a predictor of neonatal death [179] and increased risk of disability [180]. Many studies using rats HIE models described the animal conditions based on the 5 criteria of the Apgar score as a short term effect of HIE [168, 181, 182].

To establish the inflammatory status of pup brains, brain samples were collected at 1, 2 and 6 hours after birth then analysed using qPCR and Western blotting. HIF-1α protein expression in the pup brain was measured using Western blotting. To detect apoptosis, pup brain samples were collected at P1, 3 and 7 and analysed using immunofluorescence staining, Western blotting and TUNEL staining. To determine the possible lesion in the offspring brains, H&E staining was used on P3 and P60 brain samples. At P90, the long term neurocognitive outcome was tested by Morris water maze, T-maze alternation, fear conditioning and NOR tests.

3.4 Results

3.4.1 Survival rates of HIE model and short term behavioural changes after perinatal HI

The survival rates at 1 hour after delivery for the CS, 10 min, 11 min, 13 min, 14 min, 15 min and 16 min HIE groups were 98.3% (284 of 289, based on 59 surgeries), 97.5% (115 of 118, based on 21 surgeries), 87.5% (7 of 8, based on 1 surgery), 80% (16 of 20, based on 3 surgeries), 70% (6 of 8, based on 2 surgeries), 53.3% (222 of 416, based on 59 surgeries) and 37.5% (3 of 8, based on 1 surgery) respectively (Fig 3.1). No survival was observed following HI periods longer than 17 minutes (0 of 7, based on 1 surgery). As the aim of this study was to establish a clinically relevant model of perinatal HI in mice which could be used to determine possible treatments for this condition in humans, 15 minutes HI was chosen for further experiments.

Naïve and CS pups started regular breathing almost immediately after being delivered. The HI pups having asphyxia had to be stimulated intensively to start breathing and they showed a decreased respiratory frequency in the first 30 min after delivery, with sustained gasping. The skin colour of the spontaneous and caesarean delivered pups was pink, while the skin colour of pups who suffered asphyxia was pale. Moreover, spontaneous and caesarean delivered pups showed intensive vocalisation and motility from the very beginning after delivery, while vocalisation and motility decreased as the length of asphyxia increased.

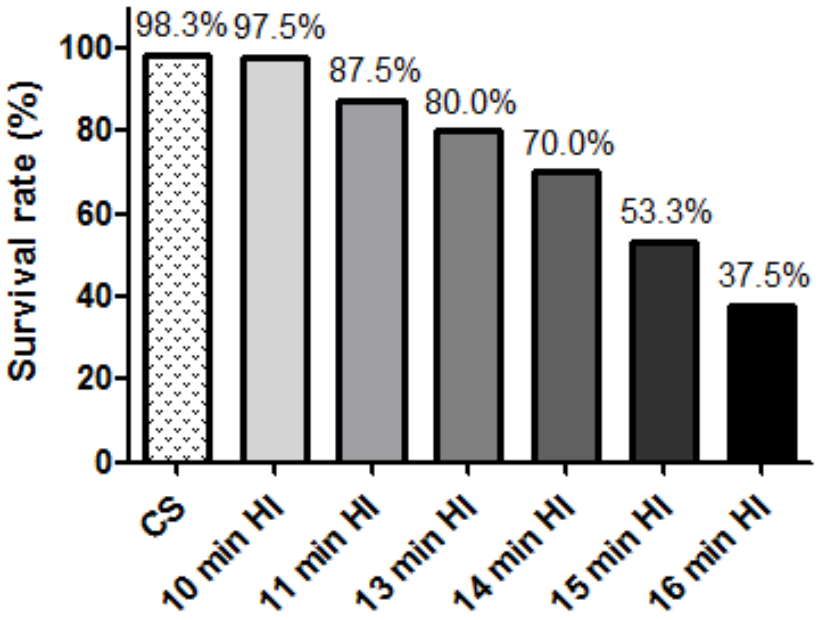


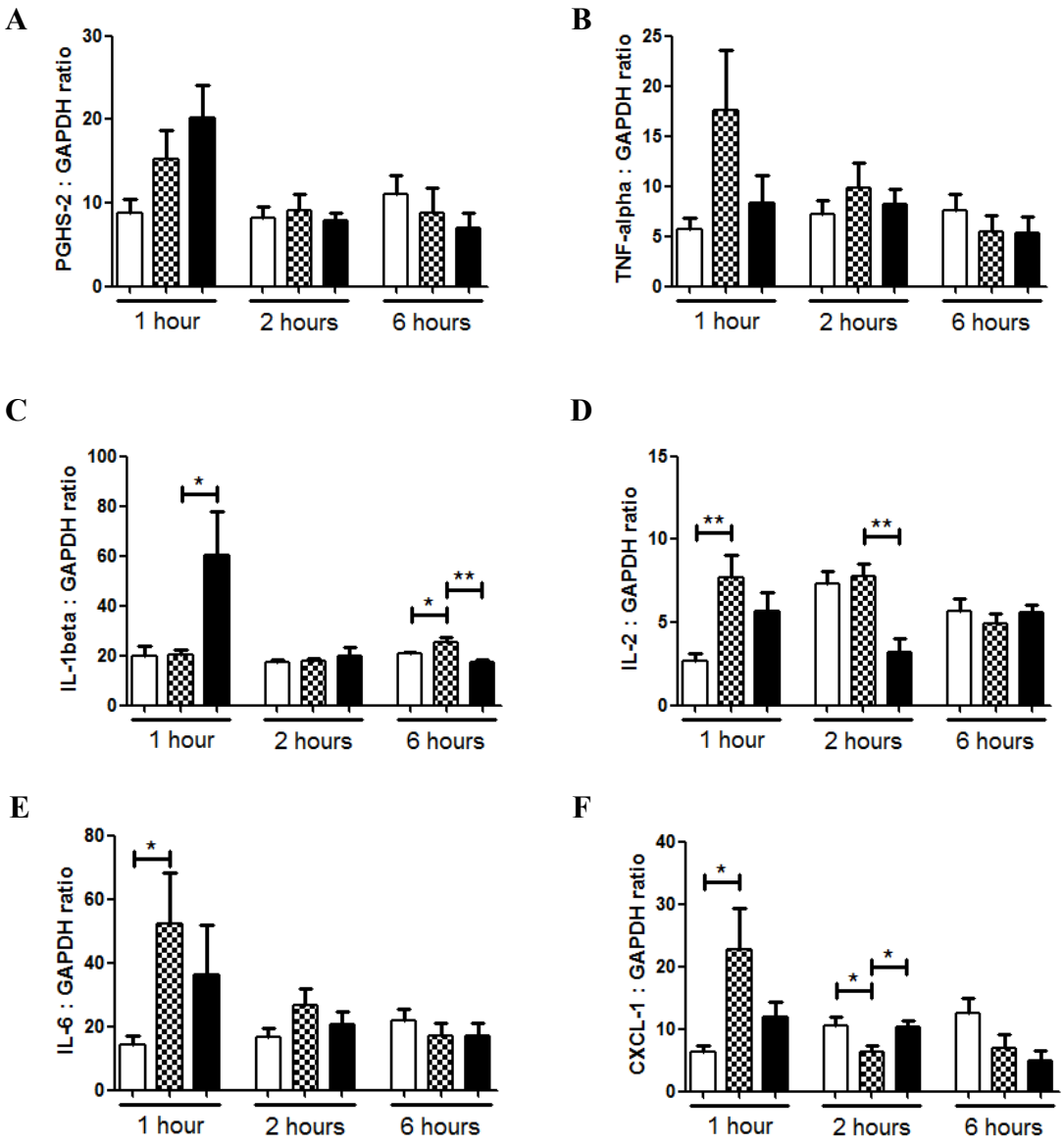
Figure 3.1. One hour survival rates of different HI time.

3.4.2 Inflammation related factors mRNA levels expressed in pup brains

Pro-inflammatory genes PGHS-2, TNF-α, IL-1β, IL-2, IL-6, CXCL-1 and CXCL-2 mRNA levels were measured in the naïve, CS and 15 min HI pup brains at 1, 2 and 6 hours after birth (Fig.3.2). There is no statistical difference between the groups in the PGHS-2 and TNF-α mRNA expression (Figs 3.2 A, B). Among several cytokines and chemokines including IL-1β, IL-2, IL-6, CXCL-1 and CXCL-2, at 1 hour after birth, only the expression of IL-1β showed significant increase in the pup brain exposed to 15 min HI compared to CS group (Fig 3.2 C). The levels of the other four cytokines were increased in the CS groups compared with the naïve groups at 1 hour after birth, but there was no significant difference between the CS and HI groups (Figs 3.2 D-G). IL-2 mRNA level showed an increased at 2 hours after birth in CS group compared to the HI group (Fig 3.2 D). Also at the 2-hour time point,

CXCL-1 mRNA level had significantly low expression in CS compared to both the naïve and HI groups (Fig 3.2 F). At 6 hours after birth, only IL-1β mRNA level showed changes between groups with levels in the CS pup brain significantly higher than in the other two groups (Fig 3.2 C).

The expression of the anti-inflammatory cytokines, IL-4 and IL-10, in pup brains was measured at 1, 2 and 6 hours after birth (Fig 3.3). IL-4 mRNA levels were significant higher at 2 and 6 hours and IL-10 at 6 hours only in the HI compared to CS group.



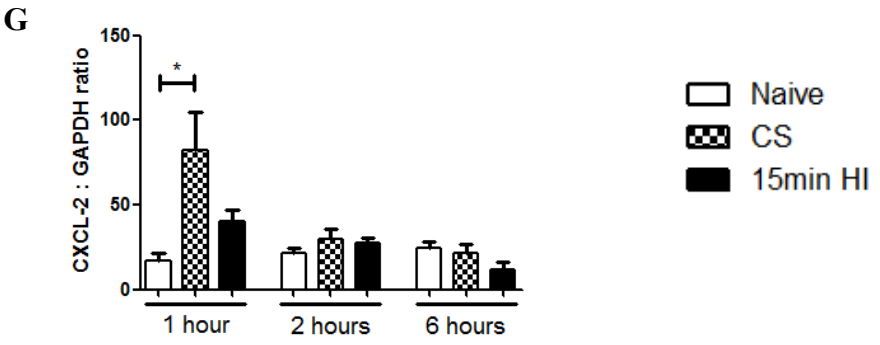


Figure 3.2. Pro-inflammatory genes mRNA levels in pup brains. Pup brains were collected at 1, 2 and 6 hours after birth. PGHS-2 (**A**), TNF-α (**B**), IL-1β (**C**), IL-2 (**D**), IL-6 (**E**), CXCL-1 (**F**) and CXCL-2 (**G**) mRNA level were measured by quantitative RT-PCR. Data were analysed using one-way ANOVA test with Bonferroni selected groups post test if the data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. n=6 in each group; *p<0.05, **p<0.01.

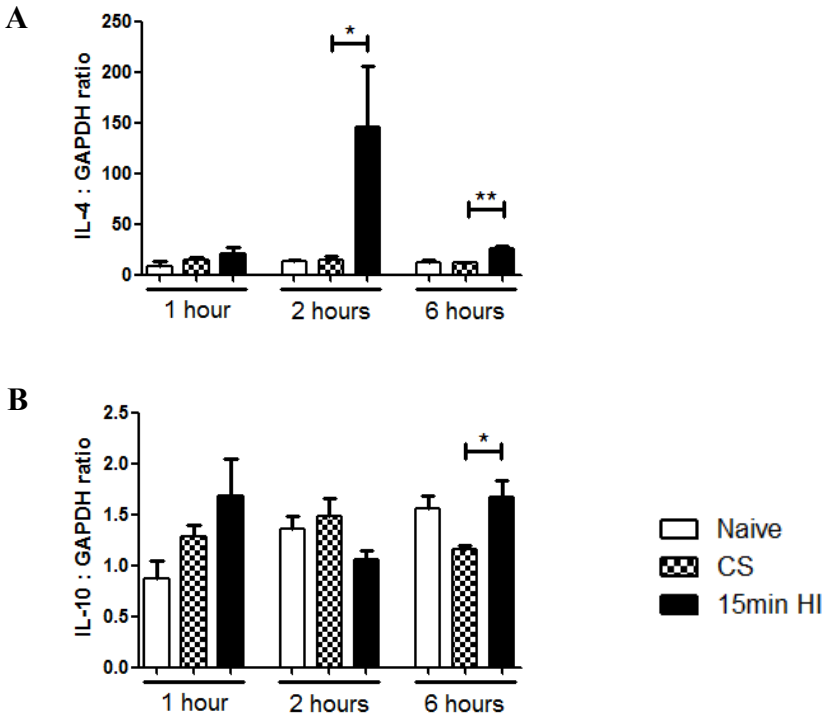


Figure 3.3. Anti-inflammatory cytokines mRNA levels expressed in pup brains. Pup brains were collected at 1, 2 and 6 hours after birth. IL-4 (**A**) and IL-10 (**B**) mRNA levels were measured by quantitative RT-PCR. Data were analysed using one-way ANOVA test with Bonferroni selected groups post test if data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. n=6 in each group; *p<0.05, **p<0.01.

3.4.3 NF-κB expression in pup brains

Activated form NF-κB p65 at different covalent modification sites (phosphorylation at Ser468 and 536 and acetylation at Lys310) and total NF-κB expression in P1, P3 and P7 pup brains were examined using Western blotting. None of them showed a positive reaction to the antibodies used. A comparison between different samples collected at various time points and a positive control (mouse myometrium sample) which was provided by a member of our research group (Dr Ektoras Georgiou) was performed (Fig 3.4) and as seen in the graph, none of the pup brain samples showed a positive result.

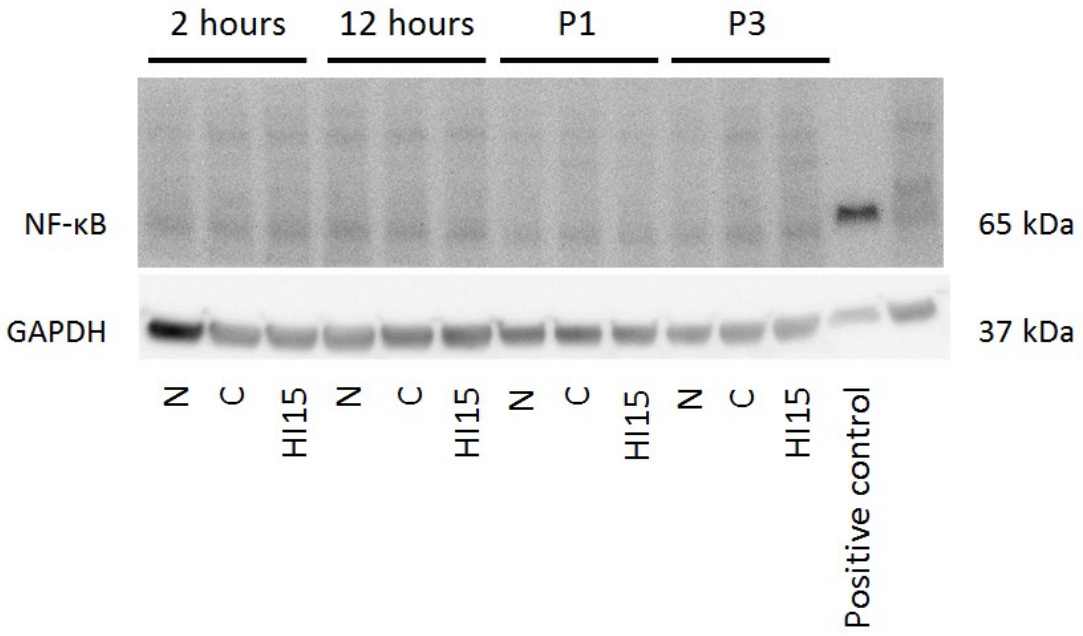


Figure 3.4. NF-κB expression in pup brain. A Western blot showing NF-κB expression in whole pup brains. The samples are taken from 3 experimental groups at various time points, post-delivery. Positive control (mouse myometrium sample) was provided by Dr Ektoras Georgiou, a member of our research group. n=1 in each group.

3.4.4 Hypoxia associated protein level expression in pup brains

HIF-α expression levels in pup brains were verified using Western blotting (Fig 3.5). No statistically significant difference between the groups was noted. However, in the normalised data set there is a trend that at P1 and P3, the HIF-1α expression is higher in the 15 minutes HI group than in all the other groups (Fig 3.5 C).

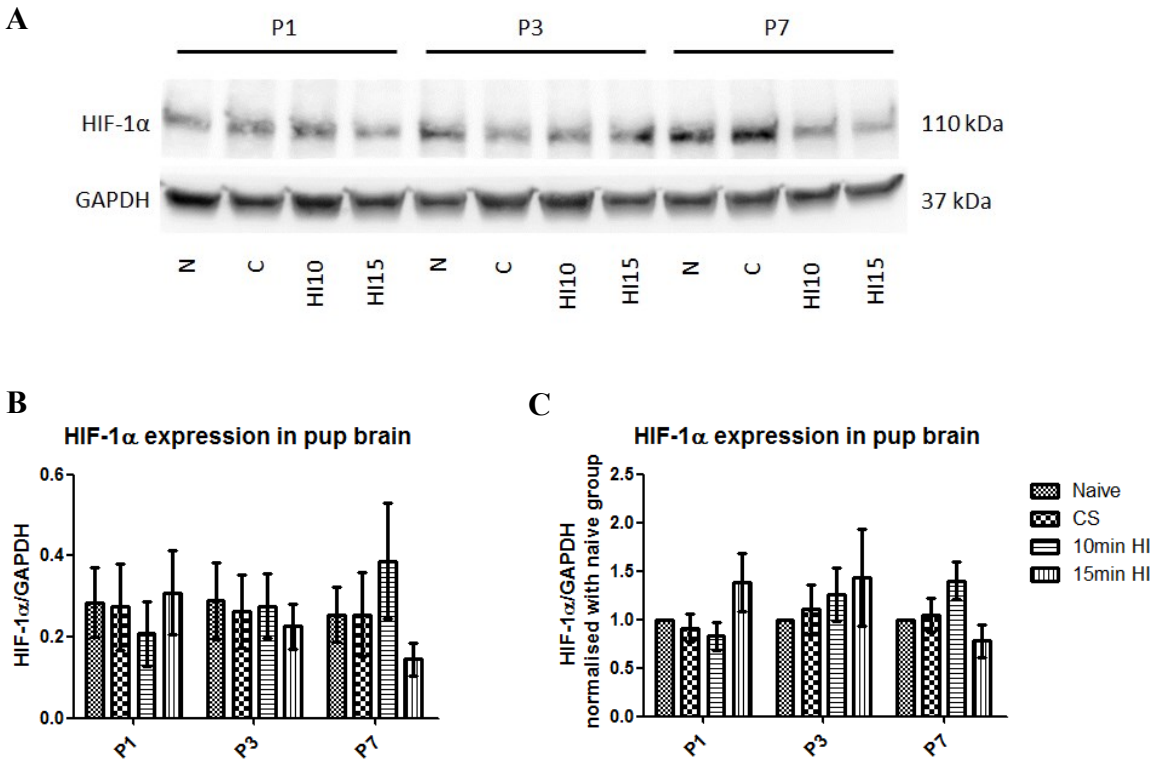
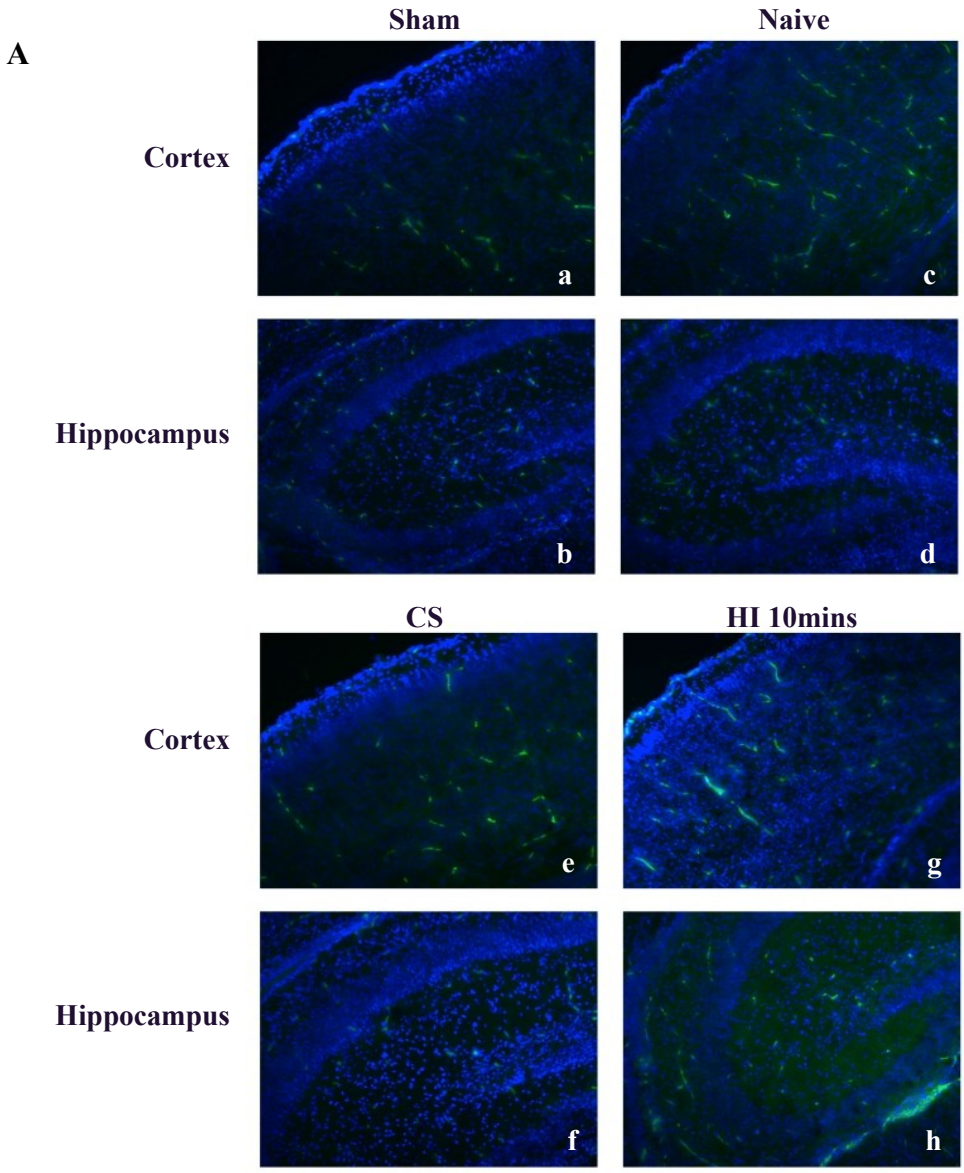


Figure 3.5. HIF-1α expression in pup brain. A. A Western blot showing varying levels of HIF-1α expression in whole pup brain taken from 4 experimental groups at 1, 3 and 7 days post-delivery. **B.** Expression of HIF-1α in naïve, CS, HI 10mins and HI 15mins groups. **C.** Expression of HIF-1α in different groups normalised with Naïve groups at P1, 3 and 7. Data were analysed using one-way ANOVA test with Bonferroni post test if the data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. n=7 in each group.

3.4.4 Apoptotic marker expression in pup brains

Active caspase-3 expression in pup brains was localised using immunofluorescence staining (Fig 3.6). No statistically significant difference between groups was observed due in part to the low n numbers and the strong non-specific staining (Fig 3.6 B). Hence, we decided to use Western blotting to quantify the expression level of caspase-3, Bax and Bcl-2.



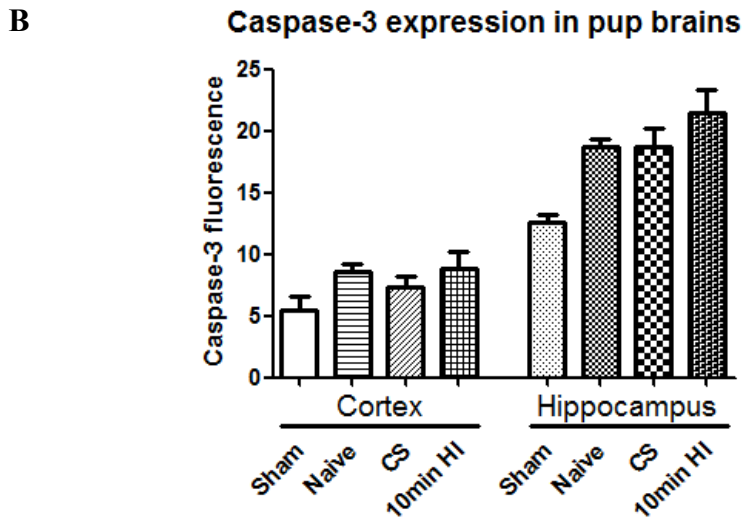


Figure 3.6. Caspase-3 expression in P3 mice pup brains. Pup brain sections (15µm) were stained with DAPI (stained for nuclei, blue) and caspase-3 (green). **A** In sham group, PBS was used to replace of caspase-3 primary antibody. Immunofluorescence images show varying levels of caspase-3 expression in the cortex and hippocampus areas of pup brains. Magnification: 100X. **B** Immunofluorescence staining of cortex and hippocampus were quantified using ImageJ software. n=3 in each group and data are presented as mean ± SEM.

Whole brain samples were used to detect global caspase-3 expression in pup brains using Western blotting (Fig 3.7 A). There was no statistically significant difference between the groups (Fig 3.7 B). Bax and Bcl-2 protein expression in the pup brains were measured using Western blotting; no statistically significant difference was observed between the groups (Fig 3.8).

The TUNEL assay is an alternative method for measuring apoptosis. In this study, apoptotic neurons were detected in the hippocampus on P7 after HI injury. Apoptotic bodies were found in CS, HI 10mins and 15mins groups, and were compared to the naïve group (Fig 3.9, arrowheads). There was no statistical difference found between the groups due to the small number of animals used.

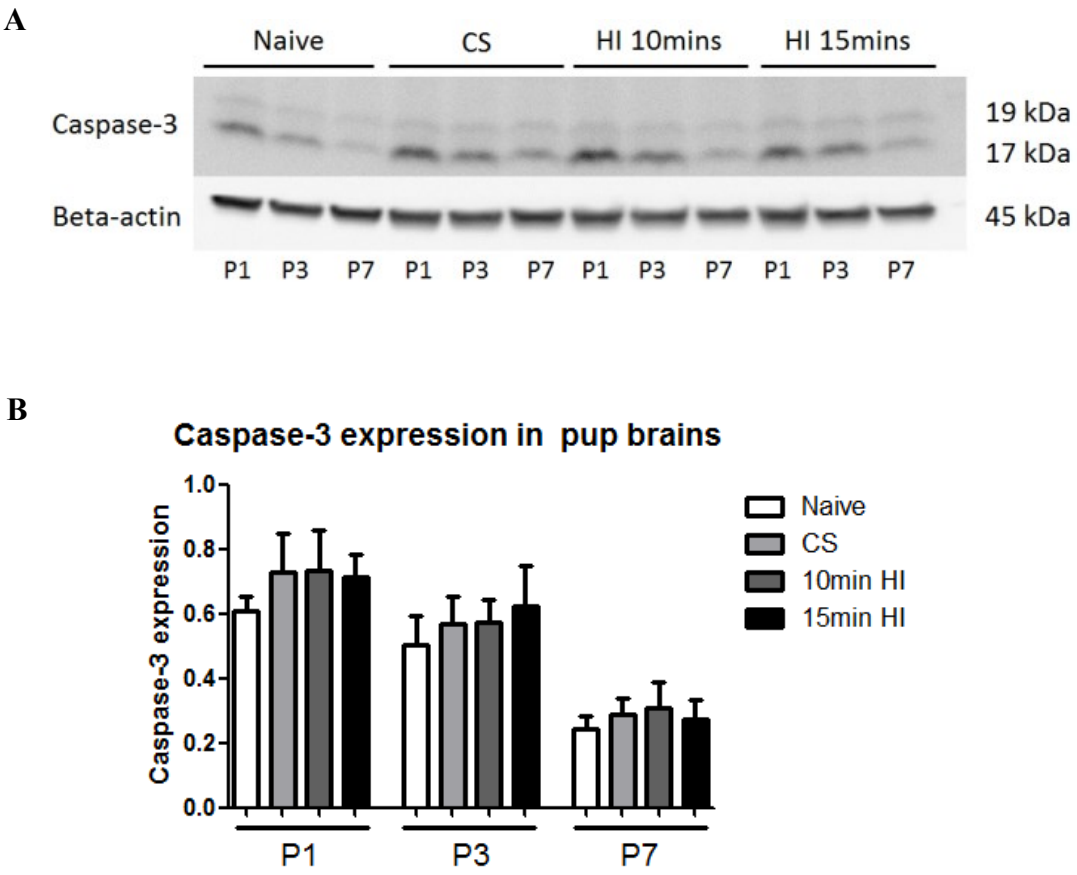


Figure 3.7 Caspase-3 expression in pup brain. A. A Western blot showing varying levels of caspase-3 expression in whole pup brain taken from 4 experimental groups at 1, 3 and 7 days post-delivery. **B.** Expression of caspase-3 in naïve, CS, HI 10mins and HI 15mins groups. Data were analysed using one-way ANOVA test with Bonferroni post test if the data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. n=8 in each group.

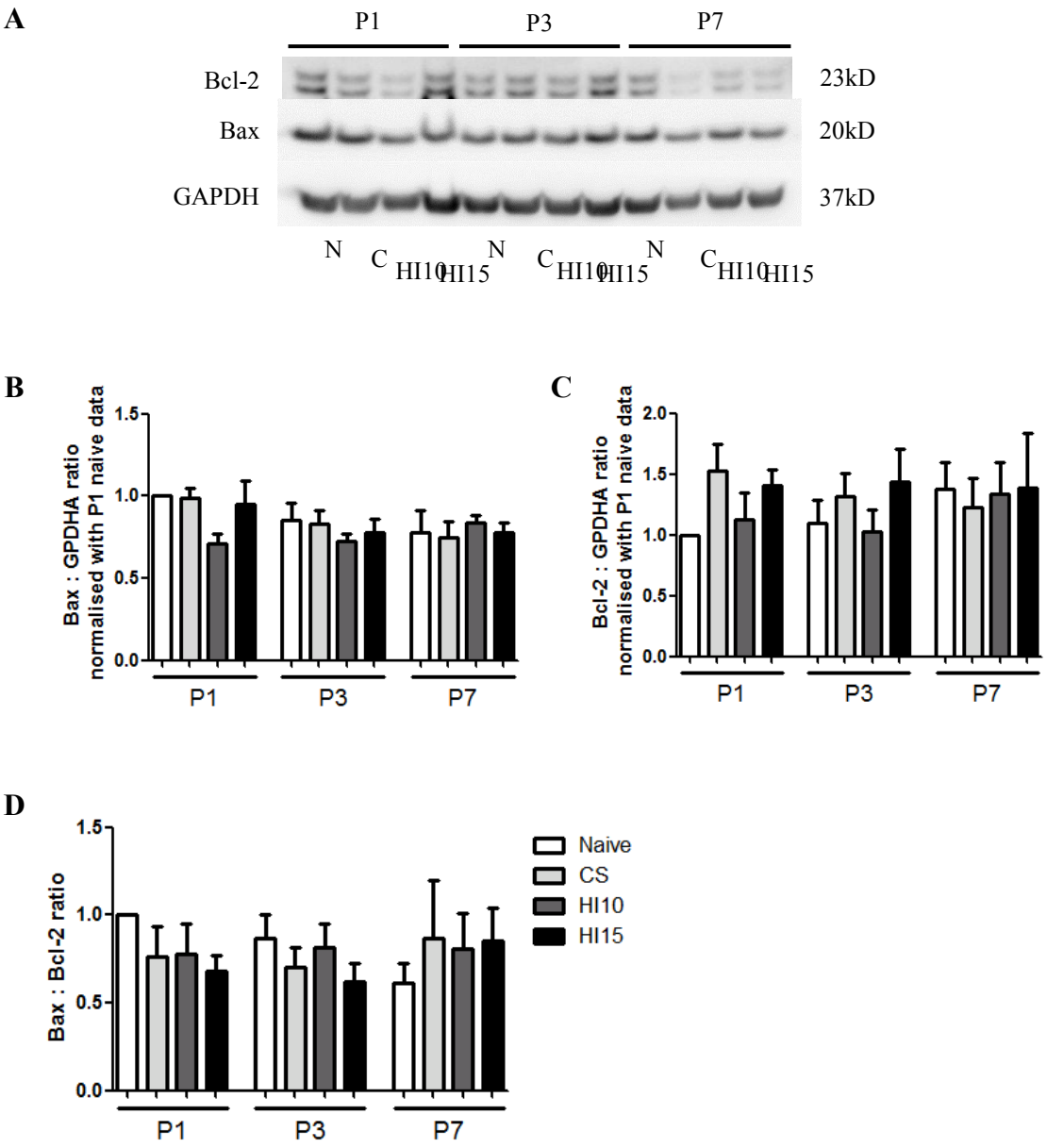


Figure 3.8. Bax and Bcl-2 proteins expression in the pup brains. Pup brains were collected on postnatal day 1, 3 and 7. **A.** Bax and Bcl-2 levels were measured using Western blotting. **B and C.** Bax and Bcl-2 data were normalised with GAPDH to exclude loading variations and then normalised with P1 naïve data to exclude gel variation. **D.** Bax and Bcl-2 ratio. Data were analysed using one-way ANOVA test with Bonferroni selected groups post test if data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. n=6 in each group; *p<0.05, **p<0.01.

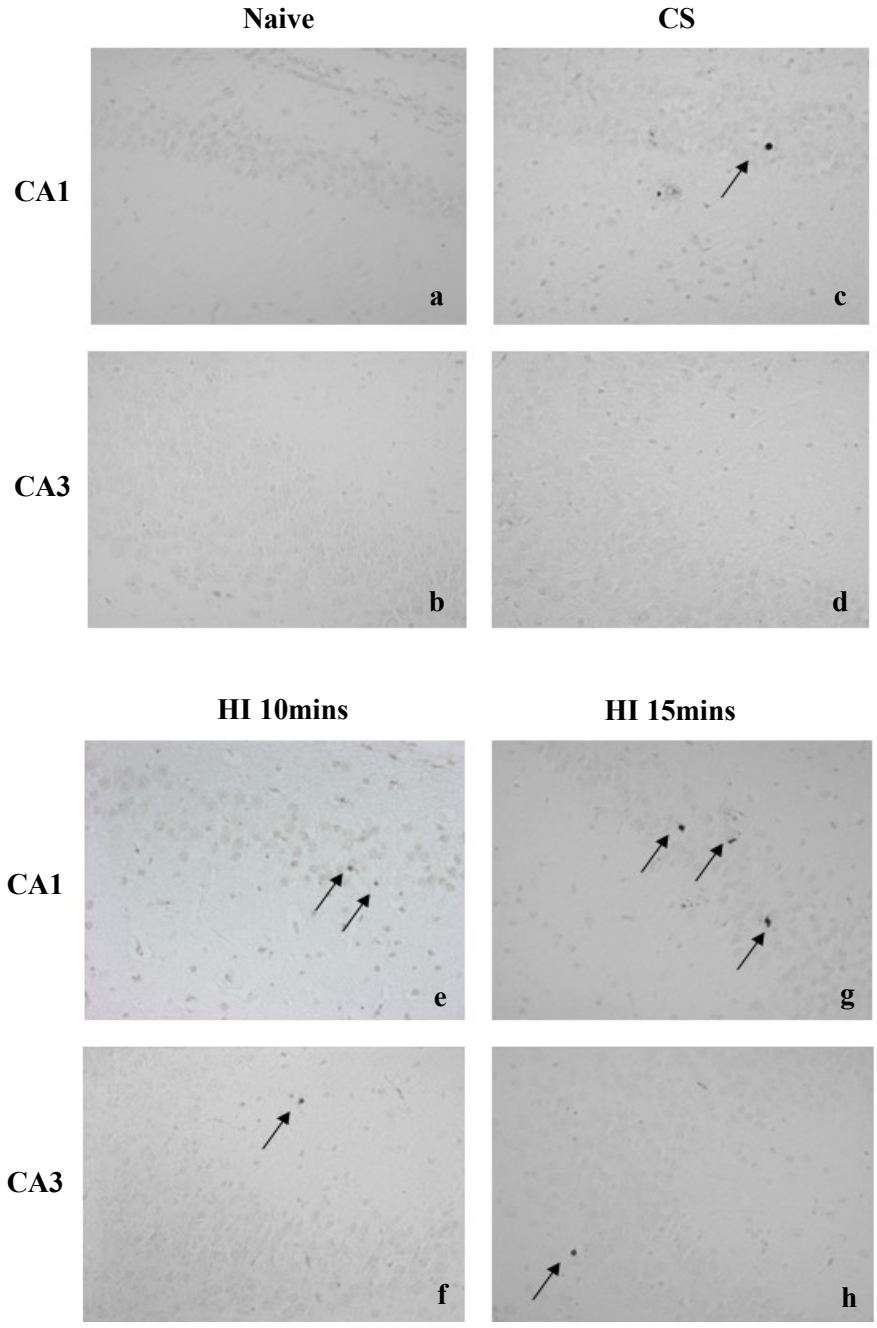


Figure 3.9 Apoptotic bodies in hippocampi of P7 neonatal mice brains
a, b, c, d. Apoptotic bodies were minimal in the CA1 and CA3 areas of hippocampus of naïve and CS control groups. **e, f, g, h.** More apoptotic bodies were seen in the two HI groups. Magnification: 200X. (n=3 in each group).

3.4.5 Immunohistochemistry

Representative photomicrographs of H&E staining show neuronal damage in the hippocampus area of the pup brains at both 3 and 60 days after birth. At P3, a number of darker stained shrunken nuclei (pointed by black arrows) appeared in both CA 1 and CA 3 areas of the hippocampus of the 15mins HI group (Fig 3.10). At P60, darker stained, shrunken, triangular shaped neurons (indicated by black arrows) were seen in both CS and 15 HI mice brains compared to the naïve group (Fig 3.11).

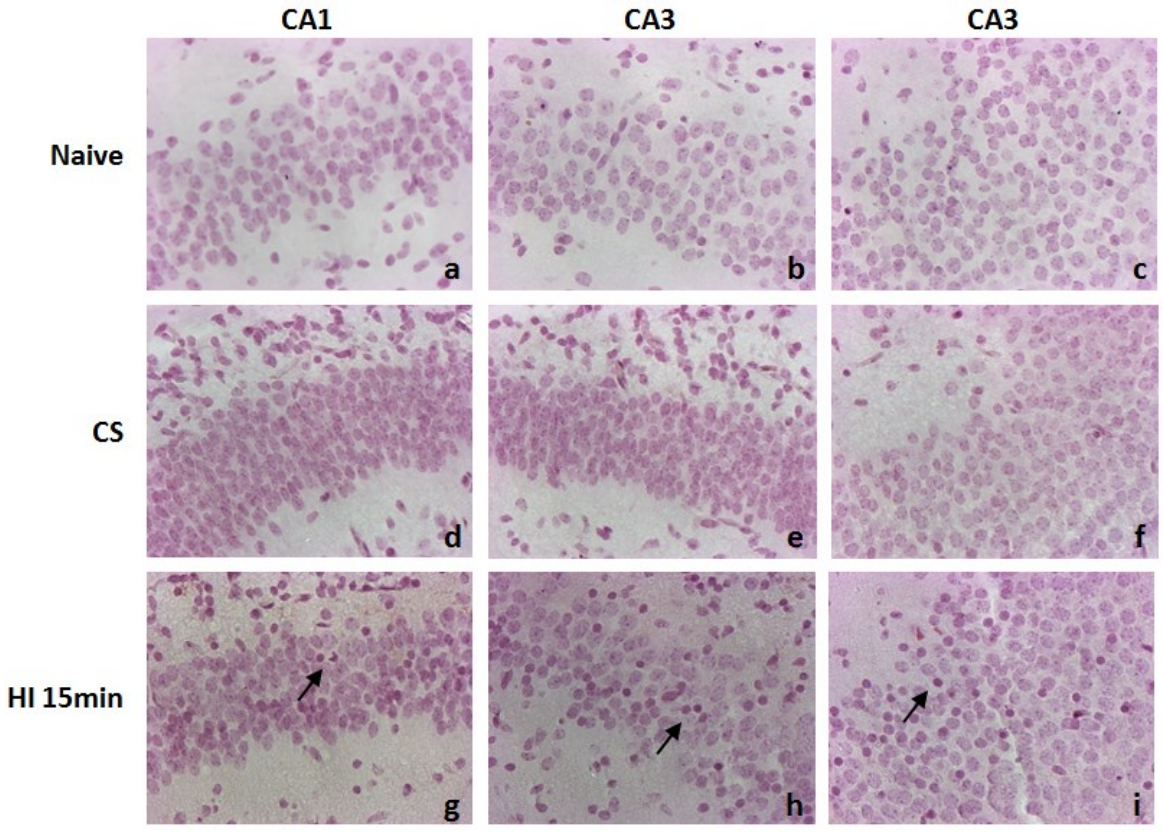


Figure 3.10. Comparison of nuclei in hippocampi of pup brain in naive, CS and HI groups. a, b, c, d, e, f. Healthy neurons in the hippocampus of naïve and CS control groups. **h, g, i.** An increased number of shrunken nuclei were found in the 15mins HI group. Black arrows indicate a few examples of shrunken nuclei. Magnification: 400X. n=3 in each group.

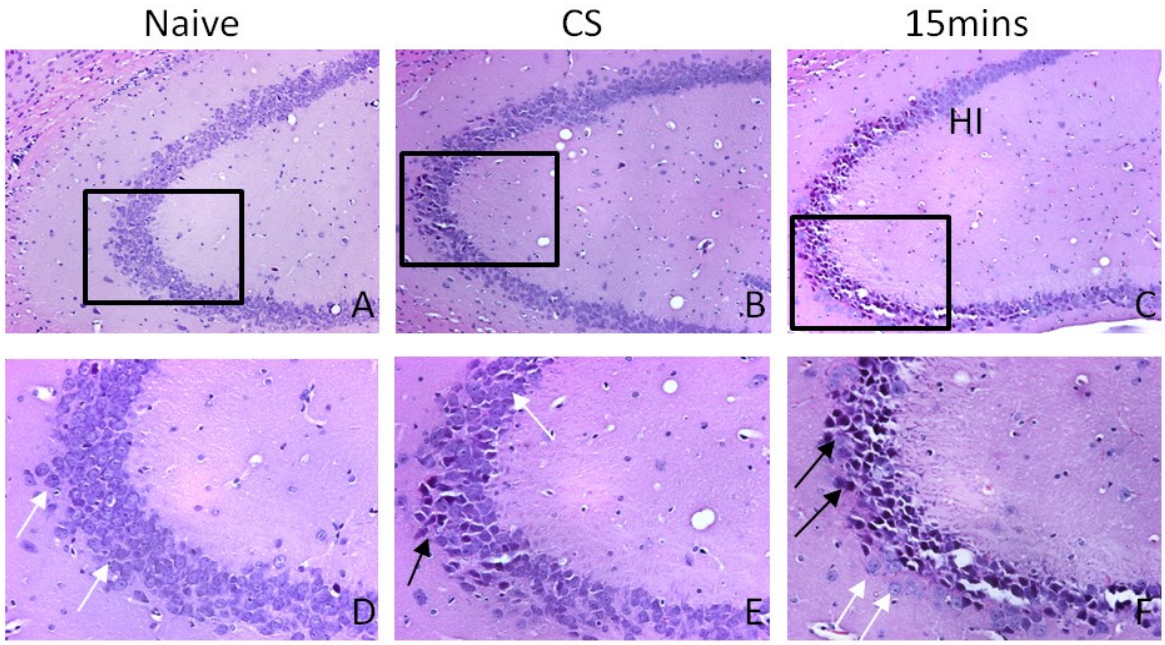


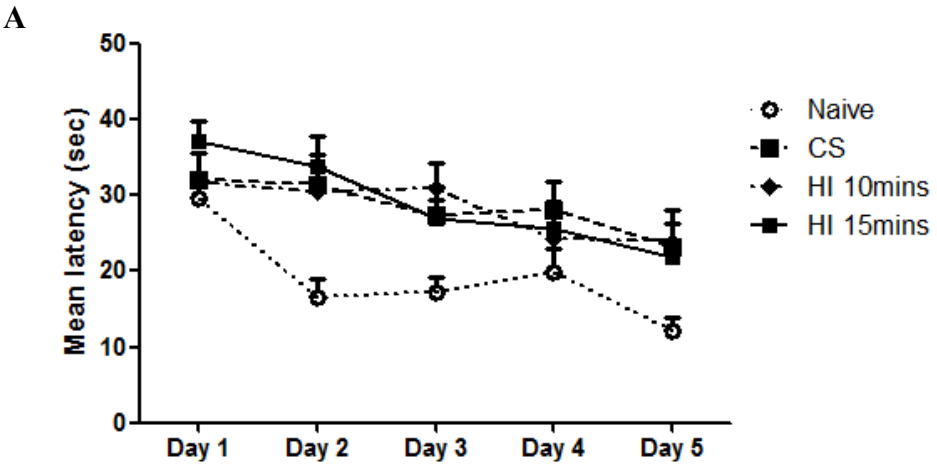
Figure 3.11 Comparison of ischaemic neurons in naive, CS and 15mins HI brain at P60. Pup brain paraffin sections (8μm) were stained with H&E. **A and D.** Healthy neurons in the hippocampus of the naïve group; **B, C, E, F.** A decrease of the total number of neurons in both CS and 15mins HI groups. The black boxes in photomicrographs A, B and C (100X) depict enlarged areas that are displayed in panels D, E and F (200X), respectively. White arrows indicate normal neurons, black arrows indicate ischaemic neurons. n=3 in each group.

3.4.6 Long-term neurocognitive outcome

The long term neurocognitive outcome was tested by Morris water maze, T-maze alternation test, fear conditioning test and NOR test.

3.4.6.1 Morris water maze

During the acquisition phase (day 1-5), latency to reach the platform was increased in animals exposed to HI compared to naïve control group (Fig 3.12 A). Area under curve (AUC; arbitrary unit) was used to indicate the cumulative time based on the 5-day training. There were significant differences when each group was compared with the naïve group (Fig. 3.12 B). In the retention phase (day 6), the data demonstrated mice in HI 10mins and HI 15mins groups spent less time in the target quadrant compared to CS control group (Fig 3.12 C). There was no difference in swimming speeds between groups (Fig 3.12 D).



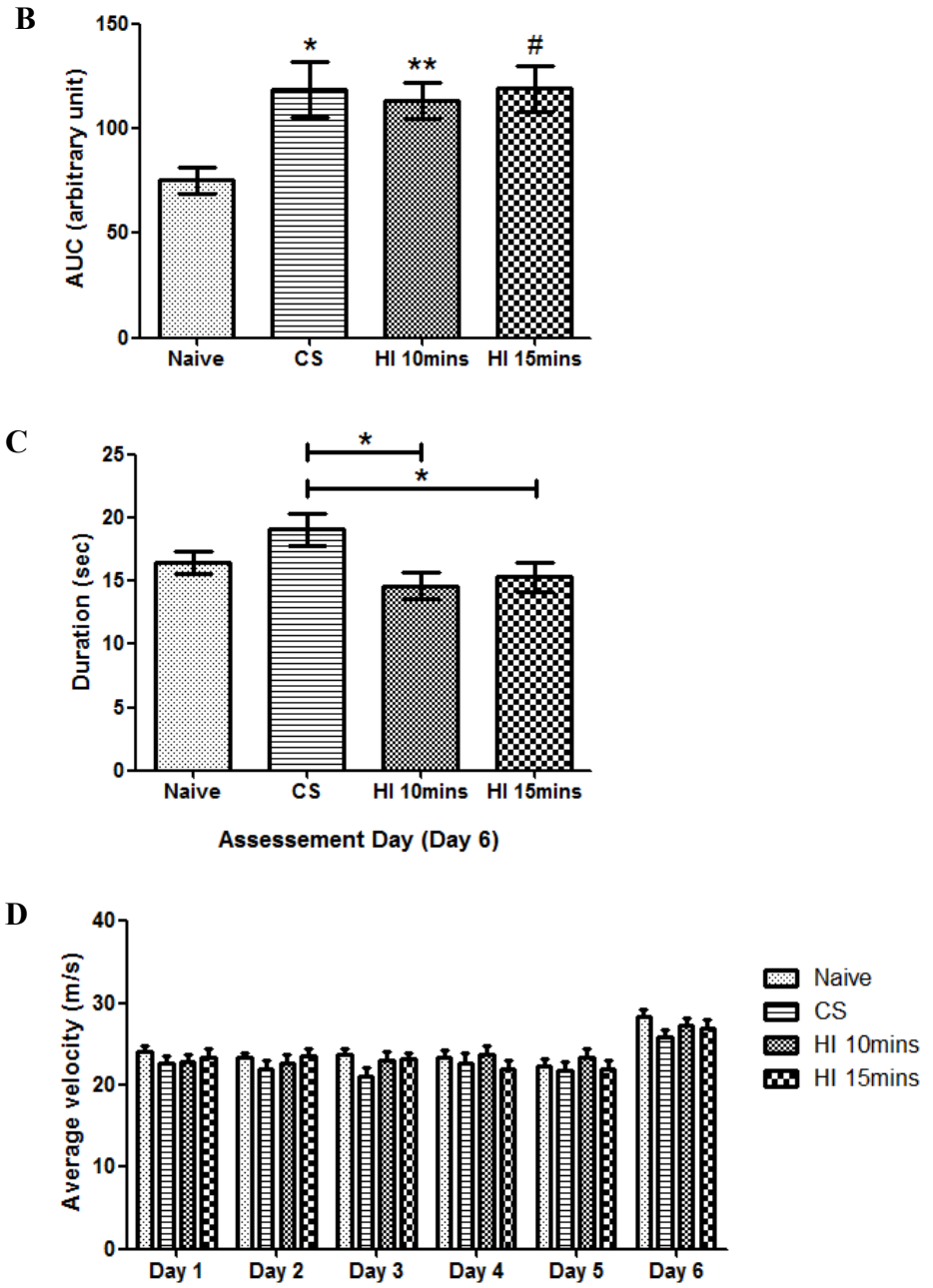
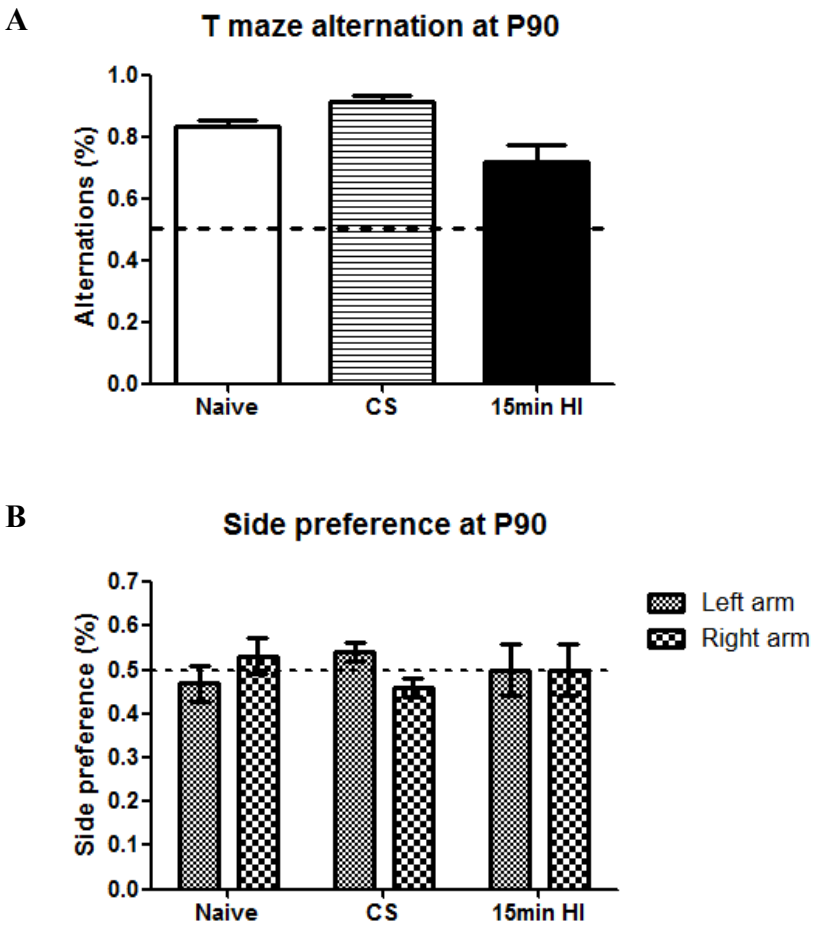


Figure 3.12. Morris water maze test performed at P45. A. The latency is defined as the time required for each animal to reach the hidden platform during five training days. **B.** The area under curve (AUC) derived from A. * CS vs Naïve $p<0.01$, ** HI 10mins vs naïve $p<0.001$, # HI15mins vs naïve $p<0.01$. **C.** Time spent in the target quadrant on assessment day. 10-min and 15-min HI groups have significant neurocognitive impairment when compared to both control groups. *$p<0.05$. **D.** There is no statistical difference between groups in the average swimming speed. Data were analysed using one-way ANOVA test with Bonferroni selected groups post test if data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM (naïve, n=19; CS, n=15; HI 10min, n=15; HI 15min, n=15)

3.4.6.2 T-maze continuous alternation task

Working memory was assessed by performing a T-maze continuous alternation task. T-maze continuous alternation task was first performed at P90 and the sample number was low. There was a trend showing that the spontaneous alternation rate of CS group was higher than the other two groups (Fig 3.13 A). However when the sample number was then increased (performed at P45) there was no significant difference between groups (Fig 3.13 C) and in the 2 groups of mice who had been delivered by C-section they preferred to turn right then left (Fig 3.13D).



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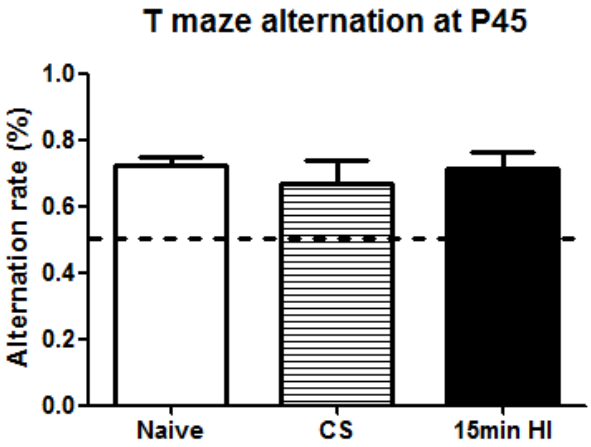

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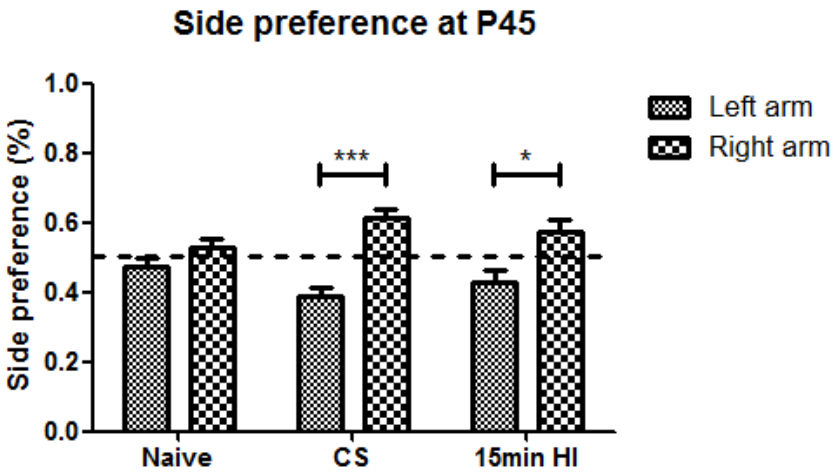


Figure 3.13. T-maze alternation at P90 and P45. A and C. The alternation rate is calculated as number of alternations/total number of choices. Dotted line indicates 50%. Data were analysed using one-way ANOVA test with Bonferroni selected groups post test if data were parametric; and Kruskal-Wallis test and Dunns selected post test if the data were nonparametric. **B and D.** Percentage of side preference. Student t-test was used in D as the data were parametric. *p<0.05; **p<0.01; ***p<0.0001. Data are expressed as mean ± SEM. P90 (A and B): naïve, n=4; CS, n=3; HI 15min, n=4; P45 (C and D): naïve, n=11; CS, n=5; 15min HI, n=6.

3.4.6.3 Fear conditioning

Fear conditioning tests contained 3 parts; the training section was done on P55 while as both of the hippocampal dependent and hippocampal independent memories were tested on P59. The mean percentage of freezing time of the animals during the training period started to increase at trial 3 (except 15min HI group) and presents an uptrend by the end of six paired conditional – unconditional stimuli (Fig 3.14 A). The data suggest that both the condition of the animals, i.e. mode of delivered and HI time, and the paired stimuli had significant effect on the reaction of the animals during the training period (Fig 3.14 A). Hippocampal memory was assessed by a contextual test. The percentage of freezing time of 15 min HI group is significantly less than the naïve group (Fig 3.14 B). Hippocampal independent memory was tested by conditional response in a novel context involving brain regions including the prefrontal cortex and amygdala. The freezing time of mice was increased in every group after being given the conditional stimulus (Fig 3.14 C). However, there was no statistically significant difference observed between the groups in the freezing time after the conditional stimulus (Fig 3.14 C).

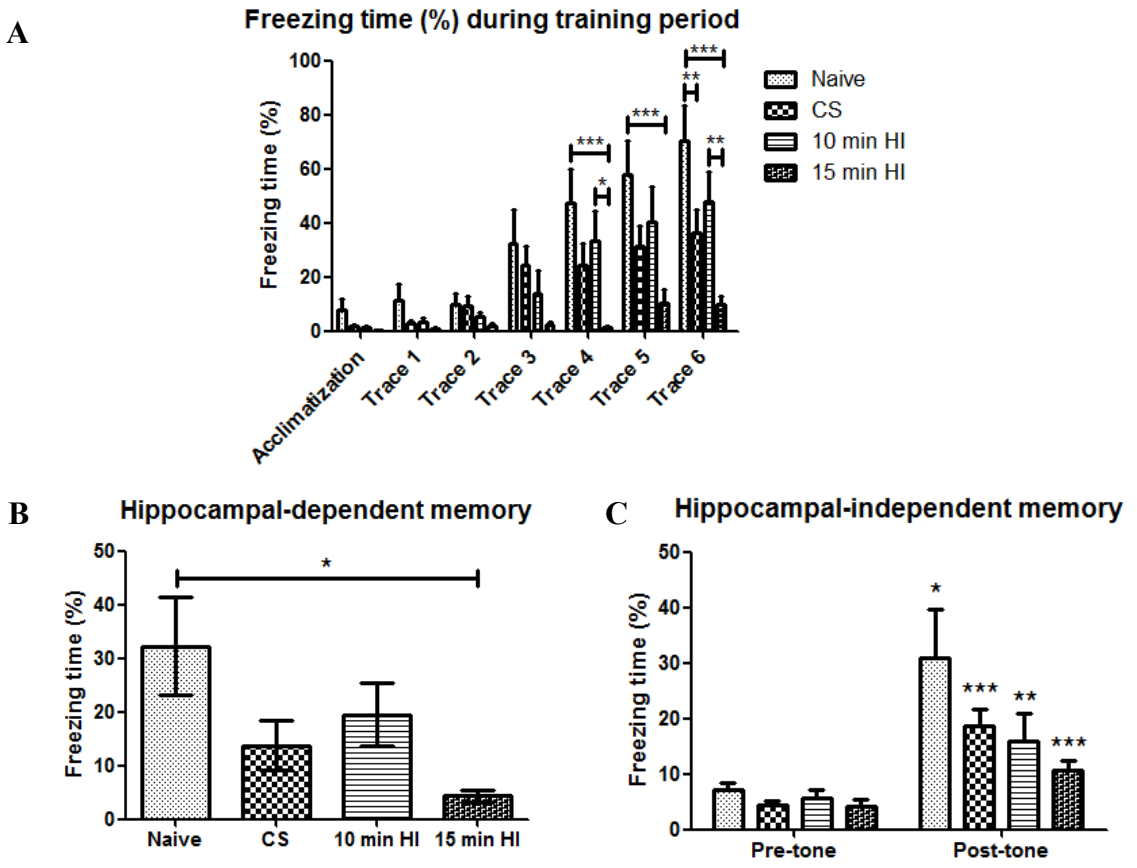
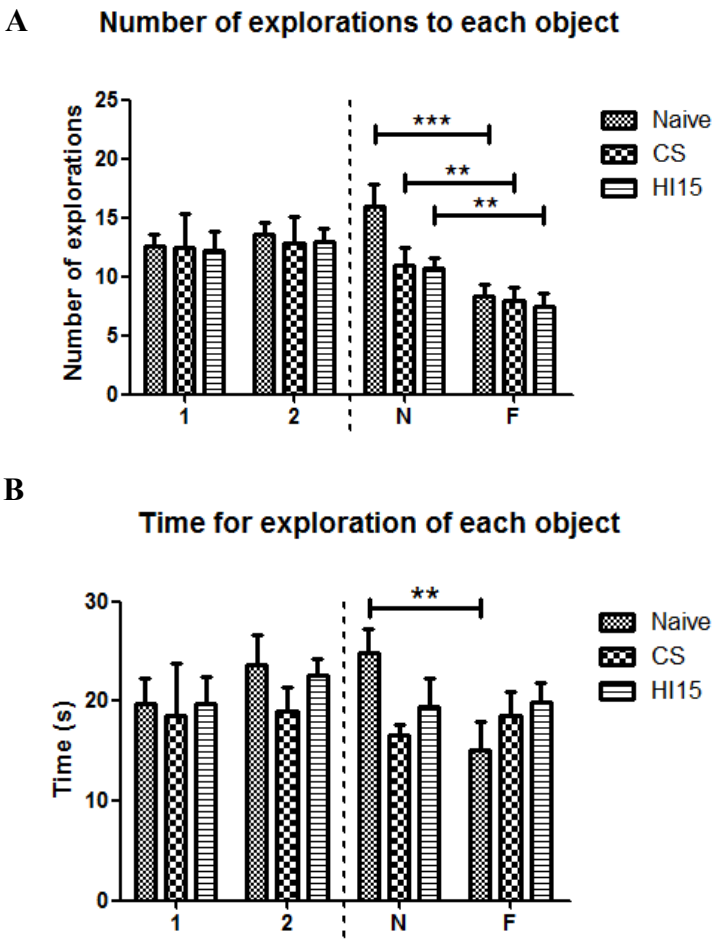


Figure 3.14. Fear conditioning test. A. The mean percentage of the freezing time after both conditional and unconditional stimuli of 4 different experimental groups in the training period. Two-way ANOVA was used followed by Bonferroni post-test. *p<0.05, **p < 0.01, *** p<0.001. **B.** The mean percentage of the freezing time during the contextual test. Kruskal-Wallis test was used and followed with Dunns post test. *p<0.05. **C.** In the conditional response test, the percentage of freezing was recorded. Student t-test was used when comparing the difference before and after the conditional stimulus was given. If data were parametric, paired t-test was used; if data were non-parametric, Wilcoxon matched pairs test was used. *p<0.05, **p < 0.01, *** p<0.001. The percentage of freezing time after the conditional stimulus was given was analysed using Kruskal-Wallis test and Dunns selected post test. Data are expressed as mean ± SEM (naïve, n=10; CS, n=19; HI 10min, n=12; HI 15min, n=9).

3.4.6.4 NOR tasks

In every experimental group, the number of explorations of the novel object was significant higher than the familiar object (Fig 3.15 A). Mice in naïve group devoted more time on exploring novel object than mice in the other two groups (Fig 3.15 B). The measure of the discrimination behaviour was the difference in the number of explorations (Fig 3.15 C) and exploration time (Fig 3.15 D) between the novel object and the familiar object. There was no statistically significant difference between the three experimental groups in the difference in the number of explorations (Fig 3.15 C). From the data of the difference of the exploration time, mice in the naïve group expressed a significantly higher discrimination index than those in the other two groups (Fig 3.15 D).



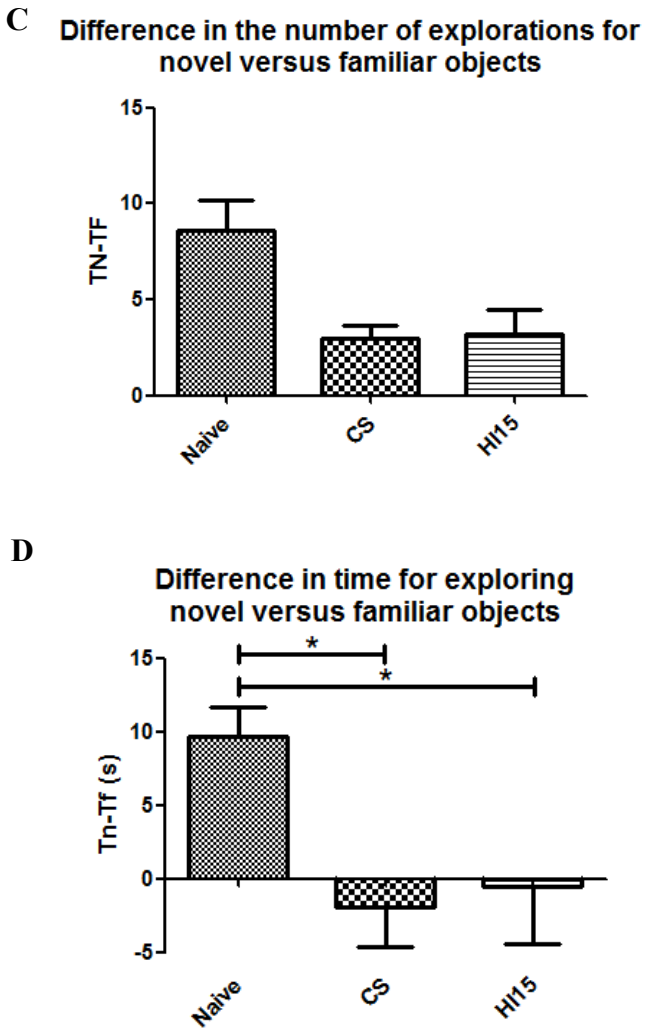


Figure 3.15. NOR test. A and B. The number of explorations (**A**) and the time spent on explorations (**B**) for each object in naïve, CS and HI 15 min groups. Data were analysed using Student t-test. If the data were parametric, paired t-test was used; if data were non-parametric, Wilcoxon matched pairs test was used. **C and D.** The difference in the number of explorations (**C**) and total exploration time (**D**). Data were analysed using one-way ANOVA test with Bonferroni selected groups post test if data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM (naïve, n=10; CS, n=5; HI 15min, n=6). *p<0.05, **p < 0.01, *** p<0.001.

3.5 Discussion

This is the first time that this perinatal asphyxia model has been established on mice. Different studies had tested different HI time (15-16, 19-20 and 21-22 minutes) on the rat model, the survival rates were around 100%, 40-80% and 5-10% respectively [168, 170, 181, 183-185]. In this study we found that CS and 10-11 minutes HI had similar survival rate at about 97-98% and 15 minutes HI in mice led to a 53.3% survival which is similar to 19-20 minutes HI in the rat model. In the present study, we found that pups exposed to 15 minutes of HI had a worse score than pups in the naïve, CS and 10 minutes HI groups when the 5 criteria of Apgar score were considered, i.e. appearance (skin colour), pulse (heart rate), grimace (reflex irritability), activity (muscle tone) and respiration. As a result of the lower survival rates and poorer short term outcomes, we chose 15 minutes of HI for establishing the HIE model and further testing of potential treatments on it.

Since the long-term cognitive dysfunction is the most significant consequence of HIE, we performed different behavioural tests to further confirm that the HIE mouse model is working or not. Morris water maze (WM) is the standard technique for assessing spatial learning and memory dependence on hippocampal functions [158]. During the training period, mice which underwent Caesarean section required a longer period to reach the hidden platform. During the probe trial the HI groups (10 and 15mins) were found to spend less time in the target quadrant than the CS group. As we did not find any difference in swimming velocity with the WM, we exclude motor deficiency as a putative factor for the poorer performance. However, in the probe trials we found that mice spent less than half of the time in the target quadrant regardless of the group studied. This might be due to the poor eye sight of CD1 mice (unpublished observations, Herbert and Johnson).

The T-maze continuous alternation task was therefore performed as another way to assess the cognitive ability of the animals. In this test, no visual cues were given as it is based on a

natural tendency to choose the remainder of 2 options, after one has been already chosen [186], this requires a working memory with a functional hippocampus [187]. In the present study, we did not find any difference between groups in the T-maze test.

Fear conditioning has become an important model for investigating the neural substrates of learning and memory in rats, mice and humans. The hippocampus and amygdala are widely believed to be essential for fear conditioning to contexts and discrete cues respectively [188, 189]. In our study, mice learnt the association between the conditioned stimulus and the unconditioned stimulus during the training period and it is noticeable that mice born naturally showed the most fear than all other groups while the mice which suffered 15 minutes of HI, showed the least. Data from FC test also indicated that mice from all groups remembered the association between the conditioned stimuli and the unconditioned stimuli. Mice born naturally linked the unpleasant experience with the contextual environment better than mice which suffered 15 minutes of HI.

The NOR tasks have become a widely used model for the investigation into memory alterations. These are particularly attractive because they require no external motivation, reward, or punishment and little training or habituation. In the current study, we found that in the sample trial while the mice were presented with two similar objects they spent almost the same amount of time with the two objects regardless of the groups they originated from. In the test trial while one of the familiar objects was replaced with a novel object, it is interesting that only mice from the naïve group devoted more time to the novel object. These data indicate that mice from the naïve group had a better working brain and short term memory than mice from the other groups. It is noticeable that the CS group had an equally bad performance when compared to mice from the HI group.

These long-tern behavioural tests results have suggested that the brains of HI and CS animals were functionally different when compared to the naïve animals. Hence we subsequently

stained the brains of P60 mice for histological evidence of brain damage. It was found that the layers of neurons in the hippocampus tended to be reduced in both CS and 15mins HI brains with a greater reduction seen in HI, where the cell loss was severe. Moreover, a number of darker stained triangular shaped neurons were found in the CA3 area of the hippocampus in HI brain. The triangular shape indicates that the neurons have undergone condensation, shrinkage and a pH change in their environment. It is noticeable that these changes also appear in a small area in the brains of pups delivered by CS. Lastly, the softness of the tissues appeared to be proportional to the degree of hypoxic ischaemic damage, i.e. the more severe the damage the more fragile the brain sections appeared to be. These findings back up the behavioural tests results mentioned previously. The unhealthy status of adult brains after HIE have not been previously reported in most of the literature where animal models of HIE were used. In recent years, it has been proposed that there is a third phase of neuronal injury after HIE which could last for months or years after the initial injury [5]. This tertiary mechanism was mentioned very briefly in Chapter 1 (1.2) as it has not been fully studied; however, it may be the explanation of the unhealthy neurons found in the P60 mice brain. As for the damage in brains and the worse long-term outcomes of CS animals, one possible explanation is that this may be due to the longer time it takes to revive the pups delivered by CS which perhaps itself resulting in mild HI damage. These findings were only observed in a small number of samples and hence did not reach statically significance; however, this would be worth exploring in the future.

Several human studies have shown that cytokines play an important role in the brain damage associated with HIE [190-192]. In the current study, the level of different pro-inflammatory and anti-inflammatory genes expression were measured and we compared the difference between the naïve and CS groups to see whether there was any effect of C-section itself and natural birth. We also compared CS and HI groups to determine the damage after introducing

HI to the pups. The data indicate that inflammation was present from an early stage in brains subjected to HI. However, there was a tendency for these genes to increase in the CS group when compared with the naïve group. This may be a consequence of the delivery method. When mice are delivered vaginally the mother is able to revive pups individually as each pup is delivered. However, it may be that in the CS delivery model, pup resuscitation is compromised as all pups are delivered at once.

The role of NF-κB activation in cerebral damage has been described in many *in vivo* and *in vitro* models of brain injury [193]. It can be protective by increasing the expression of antioxidants, growth factors and anti-apoptotic molecules. However, it can also promote neuronal death by up-regulating expression of pro-apoptotic factors such as p53 [194-196]. In the present study, no activated NF-κB signal was picked up in any of the experimental groups. Hence, neither neuronal protective nor neuronal damage promoting effects were observed. This result indicates that the NF-κB pathway did not become activated in this model of HI.

Hypoxia-inducible factors (HIFs), including HIF-1α, HIF-1β, HIF-2α and HIF-3α are members of the transcription factor family which can induce glycolysis, erythropoiesis and angiogenesis [197]. In response to the hypoxic condition, HIF-1α is up-regulated and mediates important endogenous adaptive mechanisms in order to maintain oxygen homeostasis [198]. Studies have found that HIF-1α plays an important role in HI events in the brain [199-201]. In this study, there was a trend of increasing HIF-1α protein expression during prolonged exposure to the hypoxic condition but this was not statisticlly significant.

Apoptotic cell death plays a key role in neurodegeneration after HI brain injury [163]. Caspase-3 is a validated marker of neuronal apoptosis [55]. Its activation is mediated both via extrinsic (death ligands) and intrinsic (mitochondrial) pathways. In this study we did not find any significant difference between the groups with respect to the caspase-3 activity in pup

brains. However, there the caspase-3 expression tended to correspond with the length of the hypoxia. Studies have shown that the Bcl-2 family of proteins play an important role in apoptosis signalling after neonatal HI damage [202, 203]. From the Western blotting data in the current study we did not find any statistical difference between the groups. The TUNEL assay was used to detect apoptosis by selectively targeting DNA fragmentation [204]. This technique detects early-stage apoptosis by looking at the initial changes in DNA fragmentation, before the nucleus undergoes major morphological changes. In the present study, neuronal apoptosis was detected in the hippocampus after perinatal asphyxia on P7 when an increase in the number of apoptotic bodies was found in the two HI groups studied when we first performed the test. When the sample number was increased the differences were less apparent. The situation was repeated when the H&E staining was performed using P3 pup brains. A distinct trend was that there was an increase in the numbers of unhealthy nuclei and incidence of neural death in the hippocampus area could be observed in the HI groups however this was not consistent.

In conclusion, these data suggest that the protocol we used for generating hypoxia in mice might lead to neuronal damage and apoptosis in the hippocampus and that these cellular changes are associated with long-term neurocognitive abnormalities in CD1 mice. However, this is not a consistent model of HIE due to the inconsistent results.

Chapter 4. Low Dose LPS Induced Systemic Maternal Inflammation during Late Pregnancy – An Extension of the HIE Mouse Model

4.1 Introduction

As previously described, maternal inflammation can exacerbate the effects of HI [62, 63], perhaps via the release of pro-inflammatory cytokines such as IL-1β and TNF-α, through which maternal inflammation has been suggested to cause neonatal brain damage [67, 205, 206]. A retrospective population-based study carried in California showed that maternal and neonatal infections were increased in cases of cerebral palsy [207]. Therefore, many studies have designed animal models mimicking maternal inflammations by administrating of lipopolysaccharide (LPS), a cell wall structural component of most gram-negative bacteria, which activates the innate immune system through interaction with toll-like receptors (TLRs) leading to an inflammatory reaction involving oxygen free radicals and the synthesis of pro-inflammatory cytokines such as IL-1β and TNF-α [67]. Studies using intraperitoneal (i.p.) administration of LPS during late pregnancy on rats have shown increasing mRNA expressions levels of IL-1β and TNF-α and glial fibrillary acidic protein-positive astrocytes together with decreasing myelin markers in the offspring brains [208, 209]. Other studies have combined the LPS-induced systemic inflammation with HI brain damage to try and understand the mechanisms that increase neonatal brain damage [210-213]. These studies suggest that maternal inflammation in late pregnancy sensitises the neonatal brain to HI-induced damage. However, in these studies, the HI was created in postnatal day 7 pups; the pros and cons of using such models have been discussed in previous chapters. Therefore, in the present study, the short- and long-term effects of prenatal exposure to LPS induced systemic inflammation combined with perinatal asphyxia on the neonatal brain were explored.

4.2 Aims

1) To define the dosage and timing of LPS to induce systemic inflammation during late pregnancy in mice.

2) To determine whether maternal systemic inflammation worsened the outcome of neonates who had previously suffered HIE brain damage.

4.3 Hypothesis

Systemic inflammation in the latter stages of pregnancy results in a worse outcome for pups exposed to HIE.

4.4 Materials and Methods

This study was firstly carried on at one of the CBS (Central Biomedical Services, Imperial College London) facilities, in the same setting as when the model was first established. However due to unforeseen circumstances (primarily non-renewal of the project licence at the venue for the second period of the PhD project), this study was then moved and continued at BSU (Biological Services Unit, University College London).

A diagram below shows the progression of this study (Fig 4.1). Briefly, pregnant mice were randomly assigned into 4 experimental groups: 2 groups with LPS (0127:B8, Sigma-Aldrich) intravenous (i.v.) injection at the dosage of 100 (LPS 100) and 200 μg/kg (LPS 200); 2 control groups with saline i.v. injection (saline) and no injection (vehicle). Saline i.v. was originally planned to be the only control group. However, due to the re-establishment of the HIE model caused by relocation of this study, there was not enough of time to perform adequate numbers of animals for the saline group hence the vehicle control was used. The i.v. injections were performed once daily from E14 to E17 and then the HIE model was

established as described previously. Maternal lung, liver, uterus and placenta tissue samples were collected immediately after cervical dislocation for accessing the inflammatory status of the dams. To examine the inflammatory and apoptosis status of pup brains, brain samples were collected at P1 and P3 then analysed using multiplex assays. At P45, the long term neurocognitive outcome was tested by the open field test (OFT) and NOR tests. The NOR tests were performed again at P90 for 2 reasons: 1) previous data had suggested that poorer behaviour outcomes had been picked up using NOR tests at P90 rather than P45 and 2) to test the animals' long-term memory. To determine the possible lesions in the offspring brains, H&E staining was used on P90 brain samples.

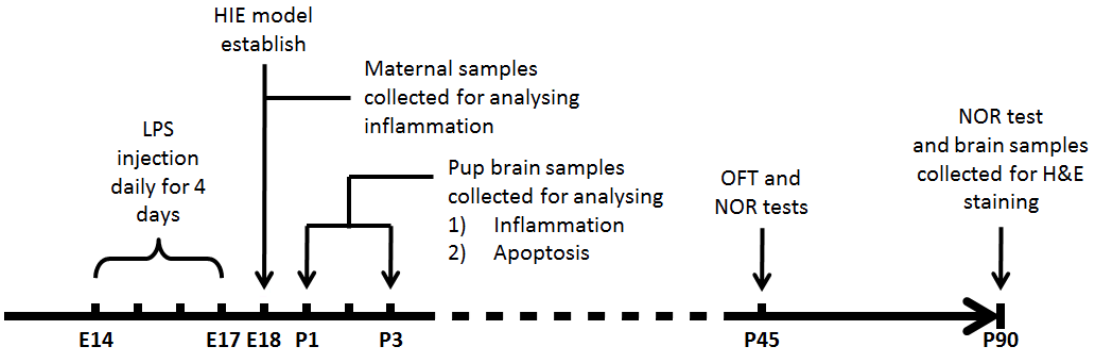


Figure 4.1. A flowchart showing the progression of this study.

4.5 Results

4.5.1 Body weights changes of pregnant mice during late pregnancy

Body weights of pregnant mice during E14 to E18 measured at two animal facilities CBS and BSU are shown in Figs 4.2 A and B. It is noticed that at both facilities in the first 24 hours after the first injection of LPS there was almost no body weights changes (Figs A and B) and these data were shown separately in Figs E and F. Among all the groups, total body weight gains at CBS and BSU experienced an upward trend (Figs 4.2 C and D). However at BSU the total weight gain after LPS 100 μg/kg injections was significantly lower than those animals that had no injection (Fig 4.2 D). It is evident that after the first 200 μg/kg LPS injection the body weight dropped significantly (Fig 4.2 E). It is also noticed that after the first 100 μg/kg injection at BSU, the body weight gain of the LPS 100 animals was significant lower than in the vehicle group (Fig 4.2 F).

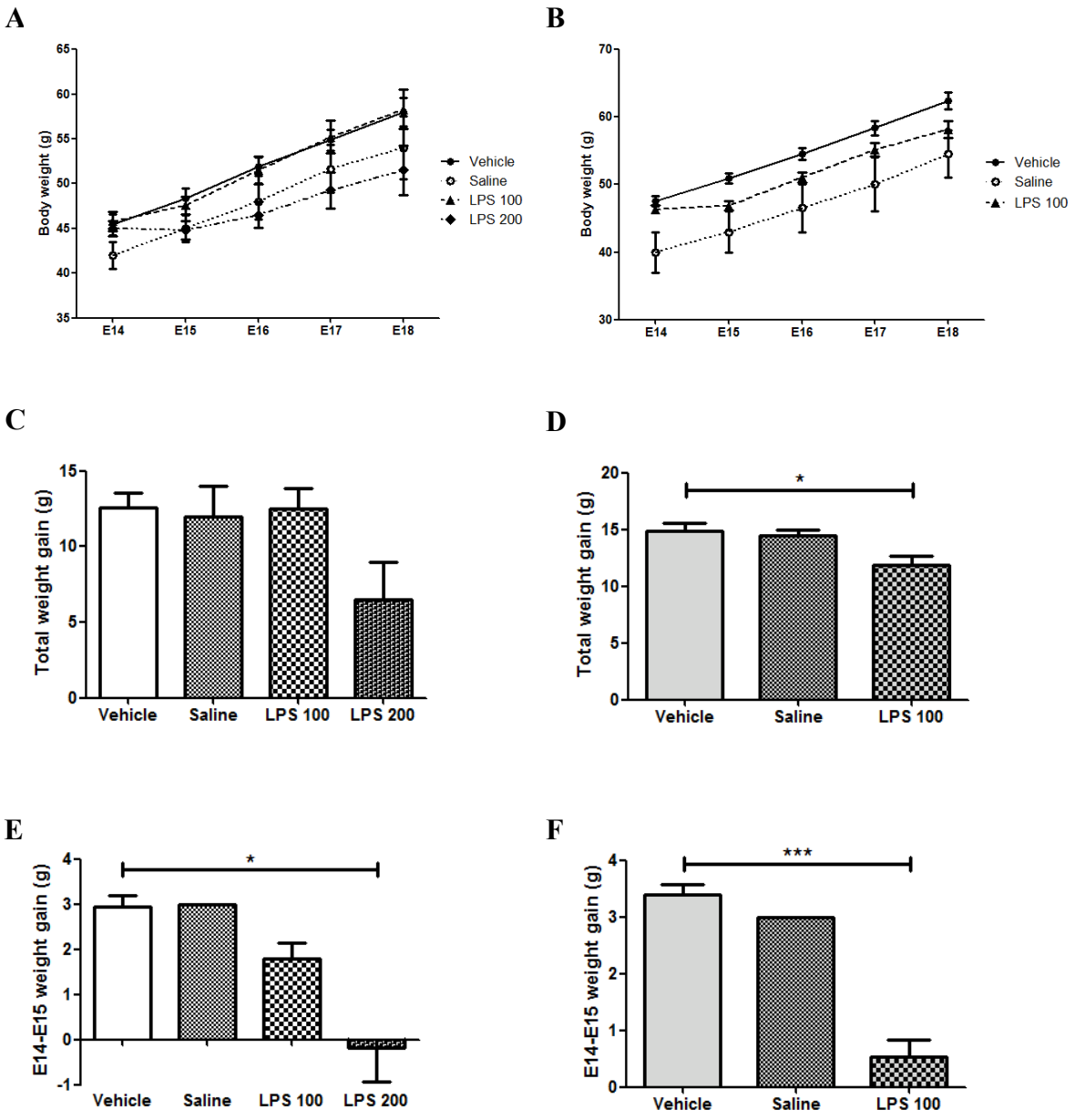


Figure 4.2. Changes in the body weights of mice at the late stage of pregnancy period (from E14 to E18). A and B. Body weights were measured daily from E14 to E18 at CBS and BSU. **C and D.** Total weight gained in the later stages of pregnancy (from E14 to E18) at CBS and BSU. **E and F.** Weight gained between E14 and E15 at CBS and BSU. Data were analysed using one-way ANOVA test with Kruskal-Wallis test and Dunns selected post test being used as the data were nonparametric. Data are expressed as mean ± SEM. CBS: no injection group n=4, saline group n=3, LPS 100 µg/kg n=7, LPS 100 µg/kg n=8; BSU: no injection group n=31, saline group n=2, LPS 100 µg/kg n=23. *p<0.05, **p<0.01, *** p<0.001.

4.5.2 Preterm birth rate, stillbirth rate and the condition of the uterus during HIE surgery

Among all the groups, only 3 mice (26 pregnant mice used in BSU LPS100 group) gave preterm birth and all the pups were dead (data not shown). The stillbirth rates in this study are shown in Fig 4.3. Data were analysed using chi-square test with Yates correction by 2 x 2 contingency table and results are shown in Table 4.1. The stillbirth rate of vehicle groups at both facilities remained at a similar low level while saline groups showed relatively large differences from site to site although this could be due to the small n numbers used. The stillbirth rate of LPS 100 was around 2.7 times higher than the vehicle group at CBS and was almost 10 times higher than the vehicle at BSU. The group that had the highest stillbirth rate was LPS 200. After considering the principles of the 3Rs (replacement, reduction and refinement) of the framework for humane animal research, the 200 μg/kg LPS maternal i.v. injection was discontinued and not used further in this study. It was noticed in certain cases that some dams which had LPS administration had a foul smell once the abdomen being opened and this was taken as a sign of inflammation.

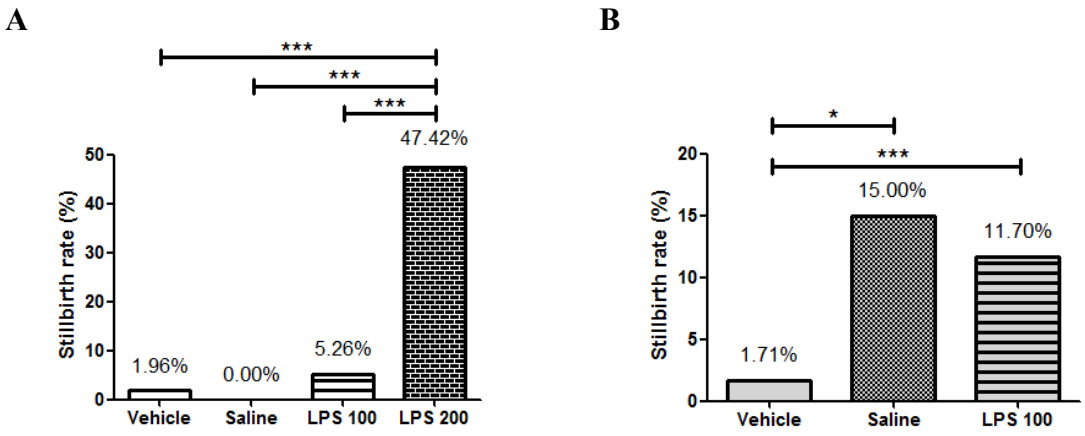


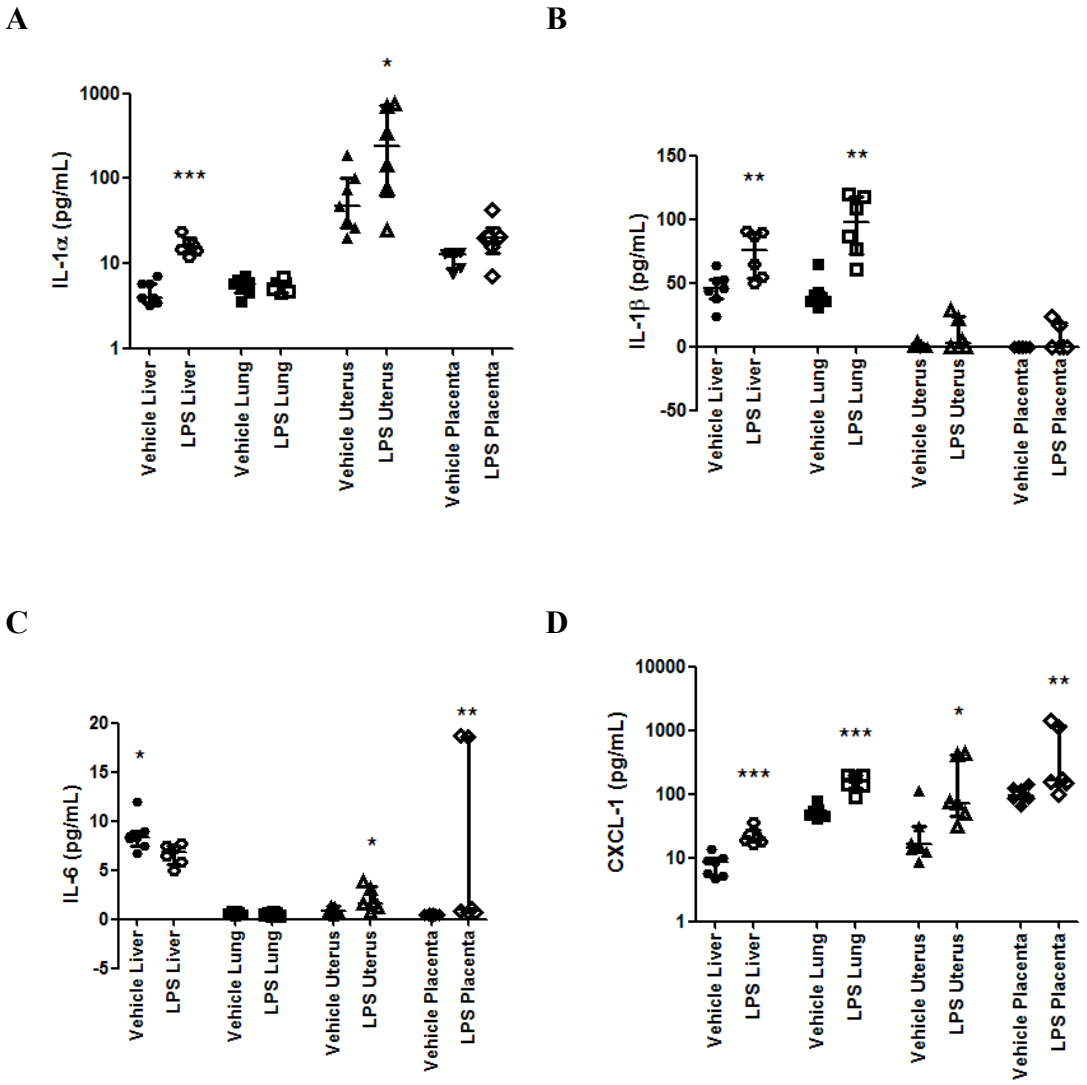
Figure 4.3. Stillbirth rates at CBS and BSU. A. Stillbirth rates of pregnant mice had no injection or after having saline or LPS i.v. injection at CBS. **B.** Stillbirth rates of different groups at BSU. Data were analysed using chi-square test with Yates correction. CBS: vehicle n=51 (4 dams), saline n=33 (3 dams), LPS 100 n=76 (6 dams), LPS 200 n=97 (8 dams); BSU: vehicle n=350 (31 dams), saline n=20 (2 dams), LPS 100 n=256 (20 dams). *p<0.05, **p<0.01, *** p<0.001.

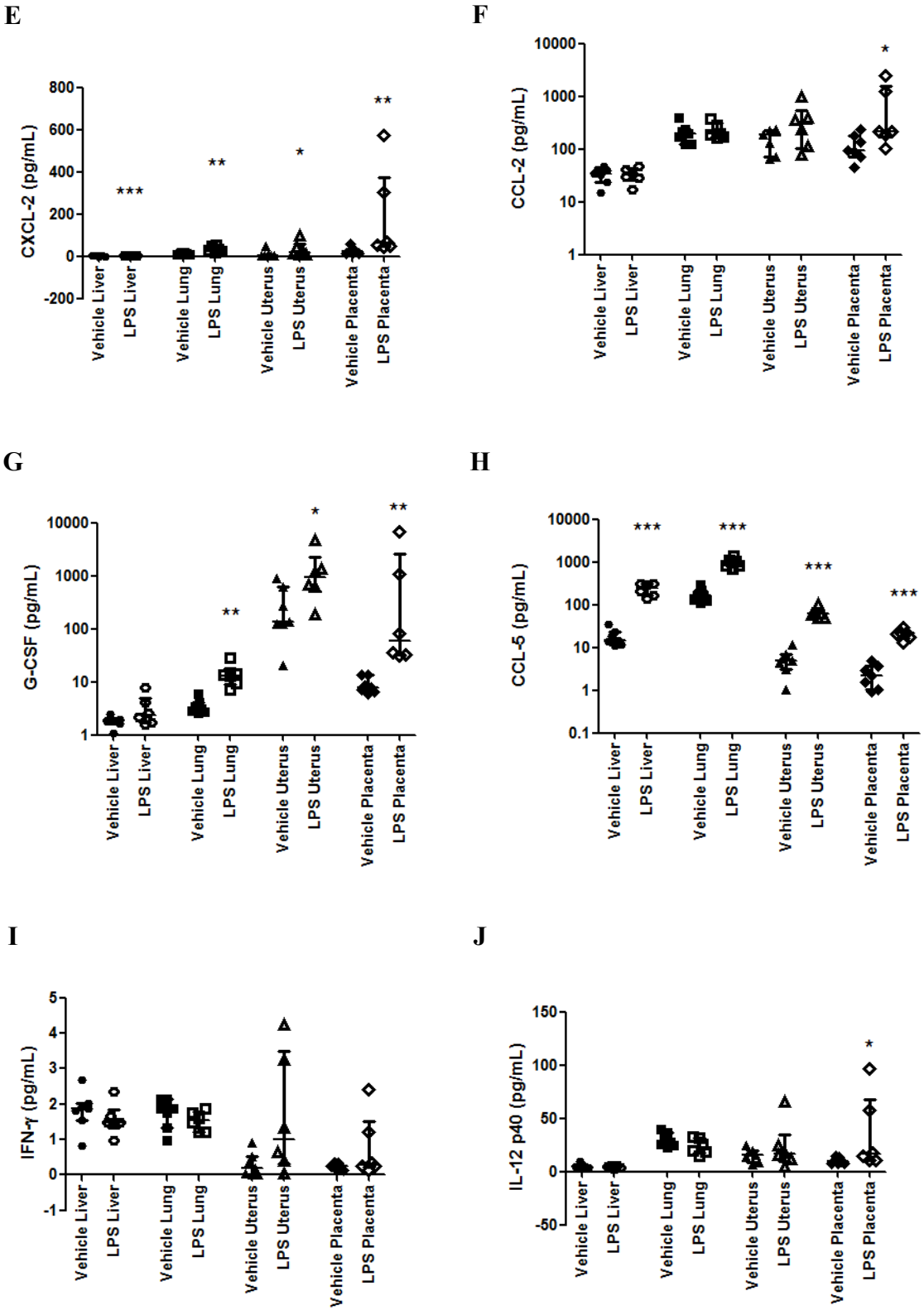
Table 4.1 Chi-square test results of stillbirth rates

CBS	Vehicle LPS 100	Chi squared 0.223 with 1 degree freedom. P=0.6364
	Vehicle vs LPS 200	Chi squared 29.814 with 1 degree freedom. P<0.0001
	Vehicle vs saline	Chi squared 0.655 with 1 degree freedom. P=0.4184
	Saline vs LPS 100	Chi squared 0.622 with 1 degree freedom. P=0.4305
	Saline vs LPS 200	Chi squared 22.190 with 1 degree freedom. P<0.0001
	LPS 100 vs LPS 200	Chi squared 34.836 with 1 degree freedom. P<0.0001
BSU	Vehicle vs LPS 100	Chi squared 23.772 with 1 degree freedom. P<0.0001
	Vehicle vs saline	Chi squared 9.03 with 1 degree freedom. P=0.0027
	Saline vs LPS 100	Chi squared 0.006 with 1 degree freedom. P=0.9380

4.5.3 Expression of inflammation-related factors in maternal tissue samples

Chemokines and cytokines expressed in maternal liver, lung, uterus and placental tissues at E18 were measured by Multiplex assays. Data are shown in Fig 4.4 individually and together in Table 4.2. As seen in the data, most of the chemokines were increase in all 4 tissues except IL-4 (Fig 4.4 M), IL-10 (Fig 4.4 N), IL-12 p70 (Fig 4.4 K) and IFN-γ (Fig 4.4 I).

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Vehicle Placenta
LPS Placenta
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G
G-CSF (pg/mL)
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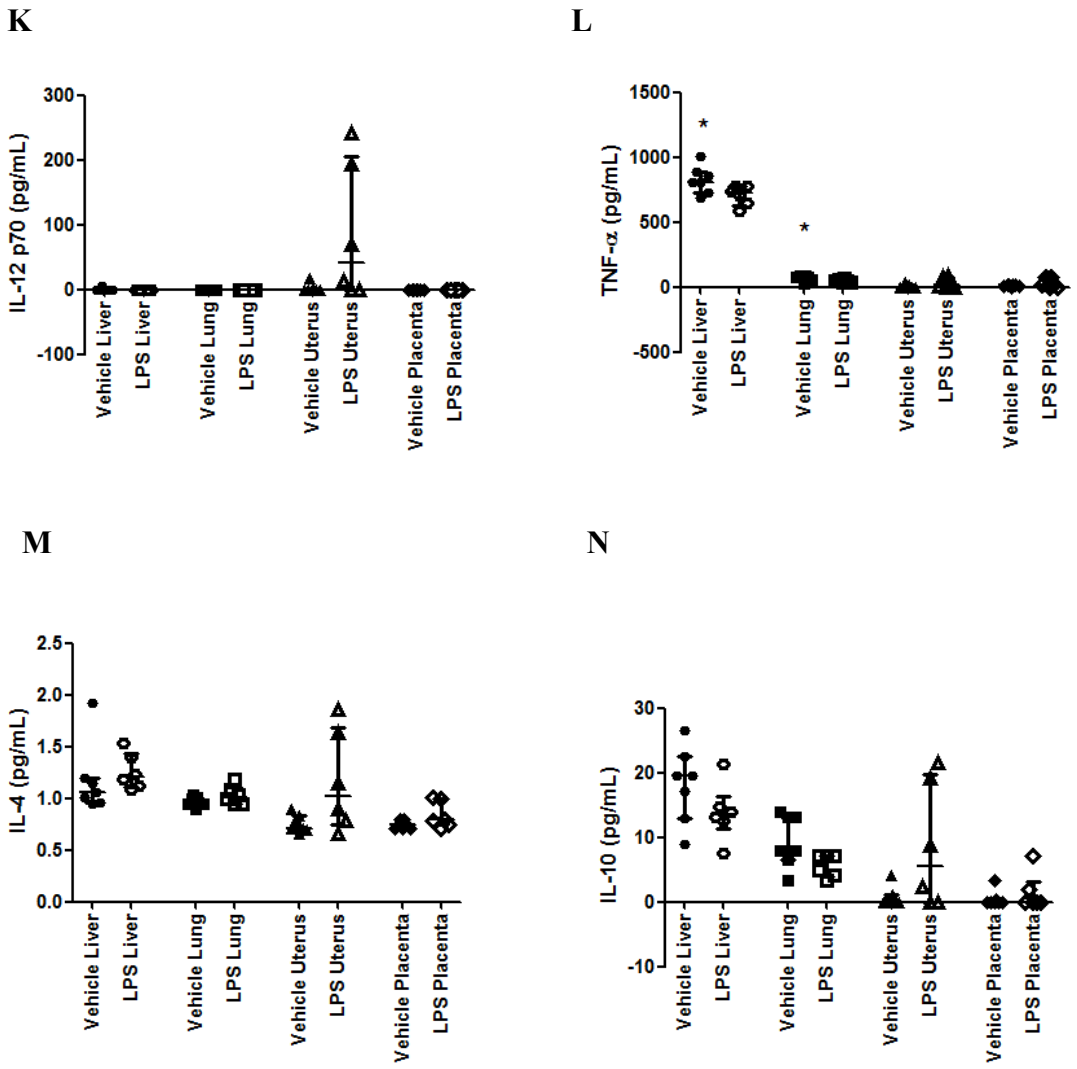


Figure 4.4. Expression of inflammation-related factor protein levels in four different maternal tissue samples. Expression of inflammation-related factor in maternal liver, lung, uterus and placenta. IL-1α (**A**), IL-1β (**B**), IL-6 (**C**), CXCL-1 (**D**), CXCL-2 (**E**), CCL-2 (**F**), G-CSF (**G**), CCL-5 (**H**), IFN-γ (**I**), IL-12 p40 (**J**), IL-12 p70 (**K**), TNF-α (**L**), IL-4 (**M**) and IL-10 (**N**) protein levels were measured by the Multiplex assay. Data were analysed using student t-test. Data are expressed as means ± SEM. n=5-6 in each group. *p<0.05, **p<0.01, ***p<0.001

Table 4.2 Changes in inflammation-related factor levels in four different maternal samples after LPS injection

Analyte	Liver	Lung	Uterus	Placenta
IL-1α	↑***	-----	↑*	-----
IL-1β	↑**	↑**	-----	-----
IL-4	-----	-----	-----	-----
IL-6	↓*	-----	↑*	↑**
IL-10	-----	-----	-----	-----
IL-12 p40	-----	-----	-----	↑*
IL-12 p70	-----	-----	-----	-----
G-CSF	-----	↑**	↑*	↑**
IFN-γ	-----	-----	-----	-----
CXCL-1	↑***	↑***	↑*	↑**
CXCL-2	↑***	↑**	↑*	↑**
CCL-2	-----	-----	-----	↑*
CCL-5	↑***	↑***	↑***	↑***
TNF-α	↑*	↑*	-----	-----

Data were concluded from the Fig 4.4. *$p<0.05$, **$p<0.01$, ***$p<0.001$. When compared to controls without LPS and the vertical arrows indicate increases and decreases.

4.5.4 Survival rates of HIE model and short term behavioural changes after perinatal HI

The 1 hour survival rates of CS and 15min HI groups after the low dose LPS administration are shown in Fig 4.5. Due to the low n number used, there is no significant difference between any of the groups (data not shown). However the much lower survival rate of vehicle group at BSU (Fig 4.5 B) compared with the one at CBS indicated that different facilities (Fig 4.5 A) may have an effect on the results of the established model. Hence the HI time to use in the HIE model was tested again at BSU. The survival rates of different HI time are shown in Fig 4.6. The survival rate after 13.5min HI was similar to the 15min HI at CBS therefore 13.5min HI was chosen for this study. The 1 hour survival rates for CS and 13.5min HI groups at BSU are shown in Fig 4.7. There was no statistically significant difference in survival rates between vehicle HI and LPS 100 HI groups. However, both the vehicle HI and LPS 100 HI were significantly higher than the saline HI, which might due to the small number of dams used in saline group.

Similar to what have been found previously, regardless of whether the dams had an i.v. injection or not, naïve and CS pups started regular breathing almost immediately after being delivered while as the HI pups had to be stimulated intensively to start breathing and they showed a decreased respiratory frequency in the first 30 min after delivery, with sustained gasping. The skin colour of the naive and CS pups was pink, while the skin colour of pups who suffered asphyxia was pale. The naïve and CS pups, regardless of which group they belonged to, were more active in vocalisation and motility from the very beginning after delivery compared to the HI pups.

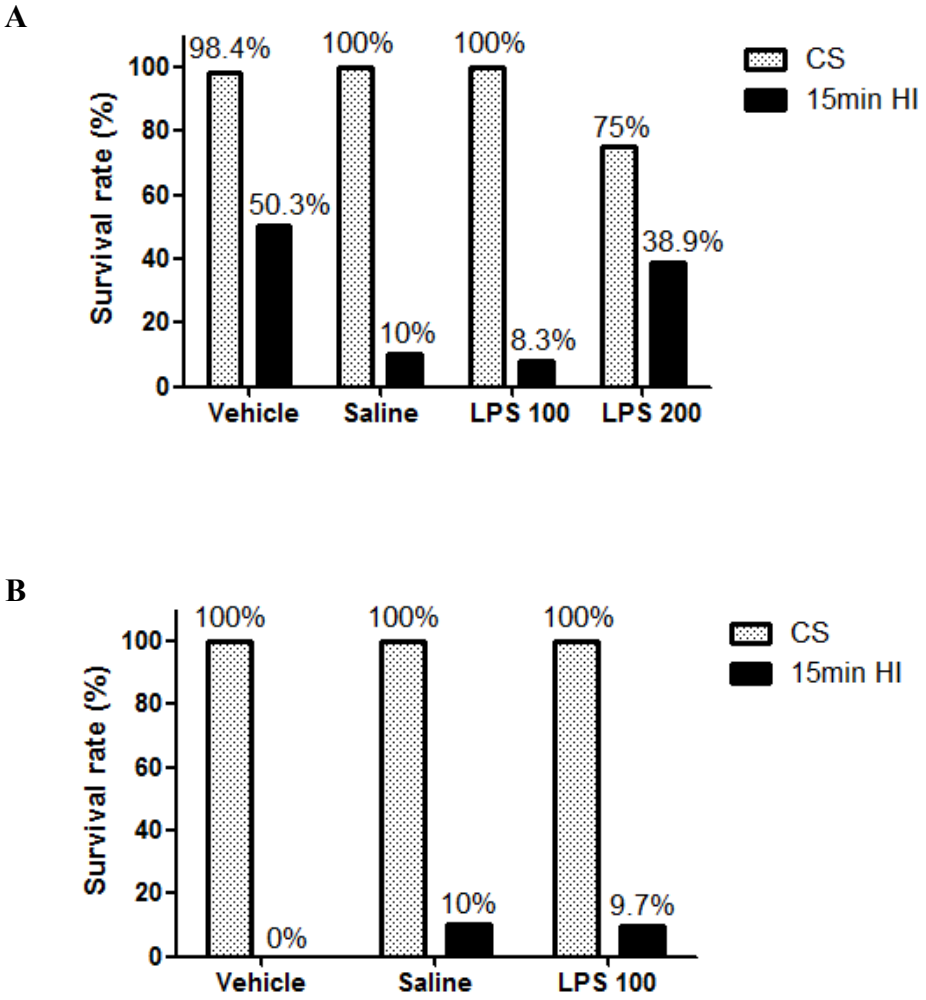


Figure 4.5. One hour survival rates of HIE at CBS and BSU. A. Survival rates at CBS. **B.** Survival rates at BSU. CBS: vehicle CS n=304 (62 dams), 15 min HI n=451 (63 dams); saline CS n=10 (2 dams), 15min HI n=10 (2 dams); LPS 100 CS n=20 (4 dams), 15min HI n=24 (4 dams); LPS 200 CS n=20 (4 dams) 15min HI n=18 (4 dams); BSU: vehicle CS n=14 (3 dams), 15min HI n=17 (3 dams), saline CS n=11 (2 dams), 15min HI n=10 (2 dams), LPS 100 CS n=18 (5 dams), 15min HI n=19 (5 dams).

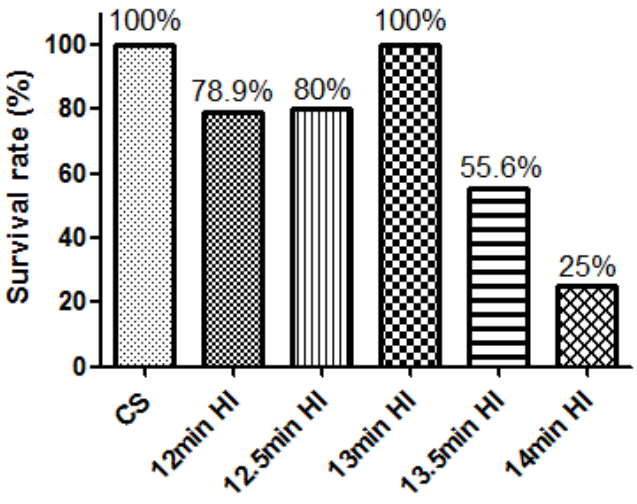


Figure 4.6. One hour survival rates of different HI time at BSU. CS n=59 (11 dams), 12min HI n=19 (3 dams), 12.5min HI n=5 (1 dams), 13min HI n=10 (2 dams), 13.5min HI n=18 (3 dams) and 14min HI n=12 (2 dams)

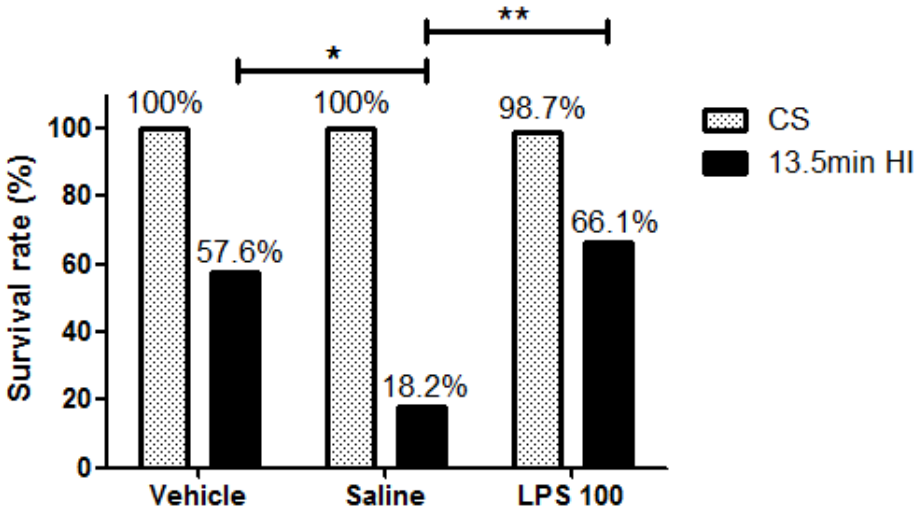


Figure 4.7. One hour survival rates of CS and 13.5 min HI groups at BSU. Data were analysed using chi-square test with Yates correction. Vehicle + 13.5min HI vs saline + 13.5min HI: p=0.0281, vehicle +13.5min HI vs LPS + 13.5min HI: p=0.2427; saline +13.5min HI vs LPS100 + 13.5min HI: p=0.0053. Vehicle CS n=80 (17 dams), 13.5min HI n=118 (19 dams), saline CS n=6 (2 dams), 13.5min HI n=11 (2 dams), LPS 100 CS n=78 (17 dams), 13.5min HI n=109 (19 dams). *p<0.05, **p<0.01, *** p<0.001.

4.5.5 Hypoxia related protein level expression in pup brains

As previously mentioned, HIF-1α, a protein whose expression in tissue, reflects an environment with insufficient oxygen, was detected by a multiplex assay system and the results for this protein are shown in Fig 4.8. At P1, the HIF-1α expression was significantly lower in the LPS100 13.5min HI group compared with the LPS100 naïve and vehicle 13.5min HI groups.

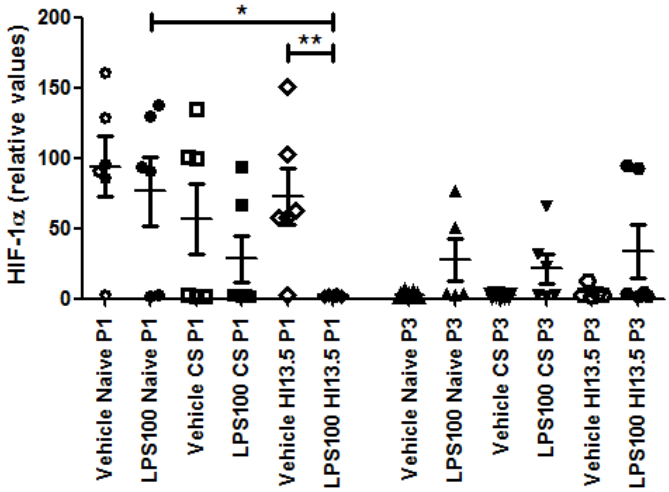


Figure 4.8. HIF-1α protein levels expression in pup brains. Pup brains were collected at postnatal day 1 and 3. HIF-1α protein levels were measured by the Multiplex assay. The fluorescence intensity (FI) value of positive control was 795.25. When comparing the effects of LPS injection on same experiment group, data were analysed using student t-test. When comparing the effects of hypoxia-ischaemia on pup brains with or without low dose LPS maternal inflammation, data were analysed using one-way ANOVA test with Bonferroni post test selected if the data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. n=5-6 in each group. *$p<0.05$, **$p<0.01$.

4.5.6 Inflammation related IL-1β and phospho-p38 protein levels expression in pup brains

IL-1β is one of the main cytokine involved in the inflammatory process when induced by LPS. P38, one of mitogen-activated protein kinases (MAPKs), participates in many cellular responses to cytokines and stress. In particular, p38 can be activated by LPS [214]. Hence in this study, to determine whether the low dose LPS administration during late pregnancy induced an inflammatory effect in the offspring, IL-1β and phospho-p38 were measured using multiplex assays. In this study, significant differences of IL-1β protein levels were only present in the two P1 naïve groups (Fig 4.9 A). The protein levels of phospho-p38 at P1 were greater in the LPS100+CS and LPS100+HI pup brains when compared with the vehicle CS and vehicle HI groups (Fig 4.9 B). In the three P1 LPS100 groups, the phospho-p38 level in LPS100+HI group was significantly higher than the LPS100+naïve group (Fig 4.9 B).

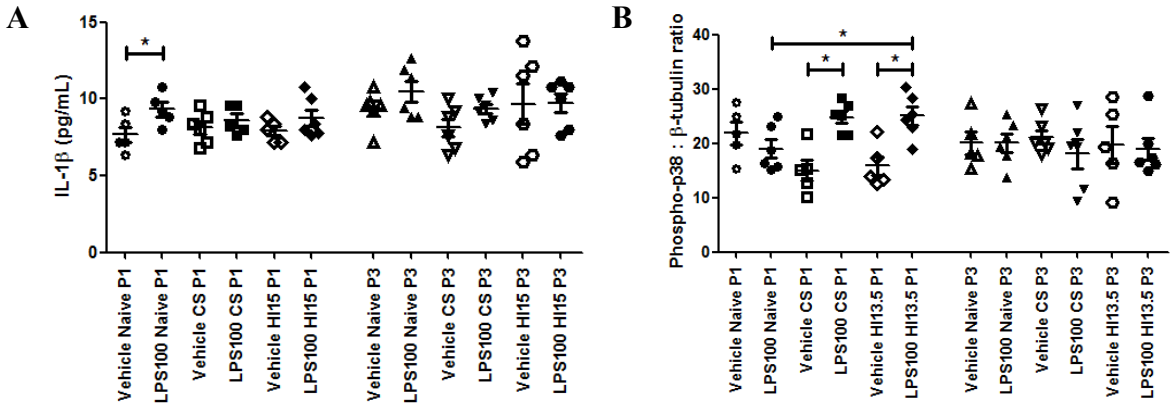
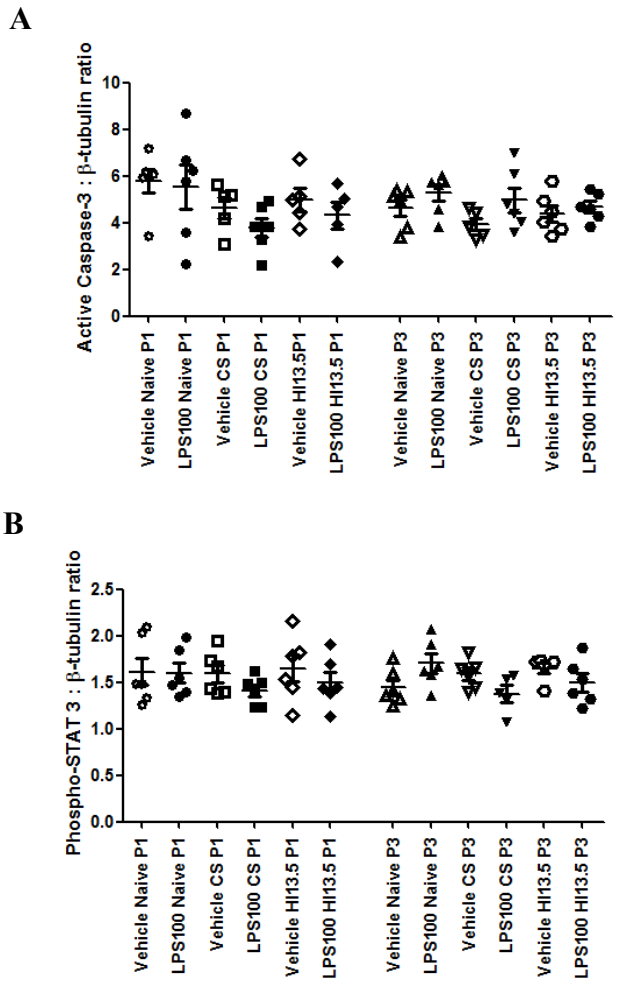


Figure 4.9. IL-1β and phospho-p38 protein levels expression in pup brains. Pup brains were collected at postnatal day 1 and 3. IL-1β (**A**) and phospho-p38 (**B**) protein levels were measured using Multiplex assay. When comparing the effects of 100µg/kg LPS and the vehicle groups, data were analysed using student t-test. When comparing the effects of hypoxia-ischaemia on pup brains after the maternal systemic inflammation, data were analysed using one-way ANOVA test with Bonferroni post test selected if the data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. n=5-6 in each group. *$p<0.05$

4.5.7 Apoptotic marker expression in pup brains

Several apoptosis related proteins expressed in P1 and P3 pup brains were detected using multiplex assays to determine whether the low dose systemic maternal inflammation had an effect on increasing apoptosis. The active caspase-3 and phospho-STAT3 protein levels remained at a similar level in pup brains regardless of the groups and time used (Figs 4.10 A and B). At P1, the expression of phospho-bad in the vehicle CS and LPS100+HI groups were significantly higher than the LPS100+CS group. At P3, the phospho-bad level was significantly lower than both the LPS100+CS and vehicle HI groups (Fig 4.10 C).



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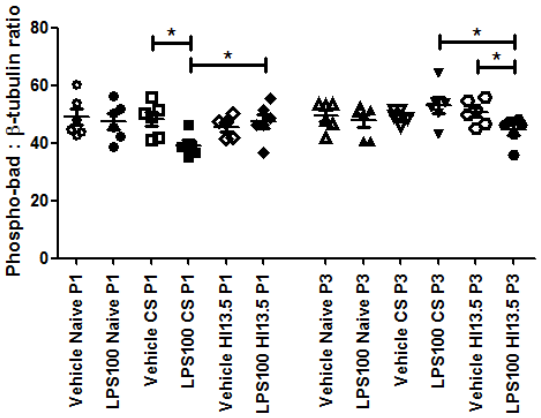


Figure 4.10. Expression protein levels of apoptosis related factors in pup brains. Pup brains were collected at postnatal day 1 and 3. Active Caspase-3 (**A**), Phospho-STAT3 (**B**) and Phospho-bad (**C**) proteins levels were measured by the Multiplex assay. Data were normalised with β-tubulin levels. When comparing the effects of LPS injection on same experiment groups, data were analysed using student t-test. When comparing the effects of hypoxia-ischaemia on pup brains with or without low dose LPS maternal inflammation, data were analysed using one-way ANOVA test with Bonferroni post test selected if the data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as means ± SEM. n=5-6 in each group. *$p<0.05$, **$p<0.01$.

4.5.8 Long-term neurocognitive outcome

The long term neurocognitive outcome was tested by the open field and NOR tests.

4.5.8.1 Open field test

During the whole 10 minutes test time, the duration in the central zone of all groups of animal was similar (Fig 4.11 A). The total travelled distance showed that among all 6 groups, the LPS100 CS group of animals travelled significant longer than the vehicle CS ones (Fig 4.11 B). To determine the fear of the animals, total freezing time of animals was recorded (Fig 4.11 C). The LPS100 naïve animals had significantly more time freezing than the vehicle

naïve animals while all the other groups of animals showed similar behaviour to each other. The mean velocity was measured and there was no significant difference between the groups (Fig 4.11 C).

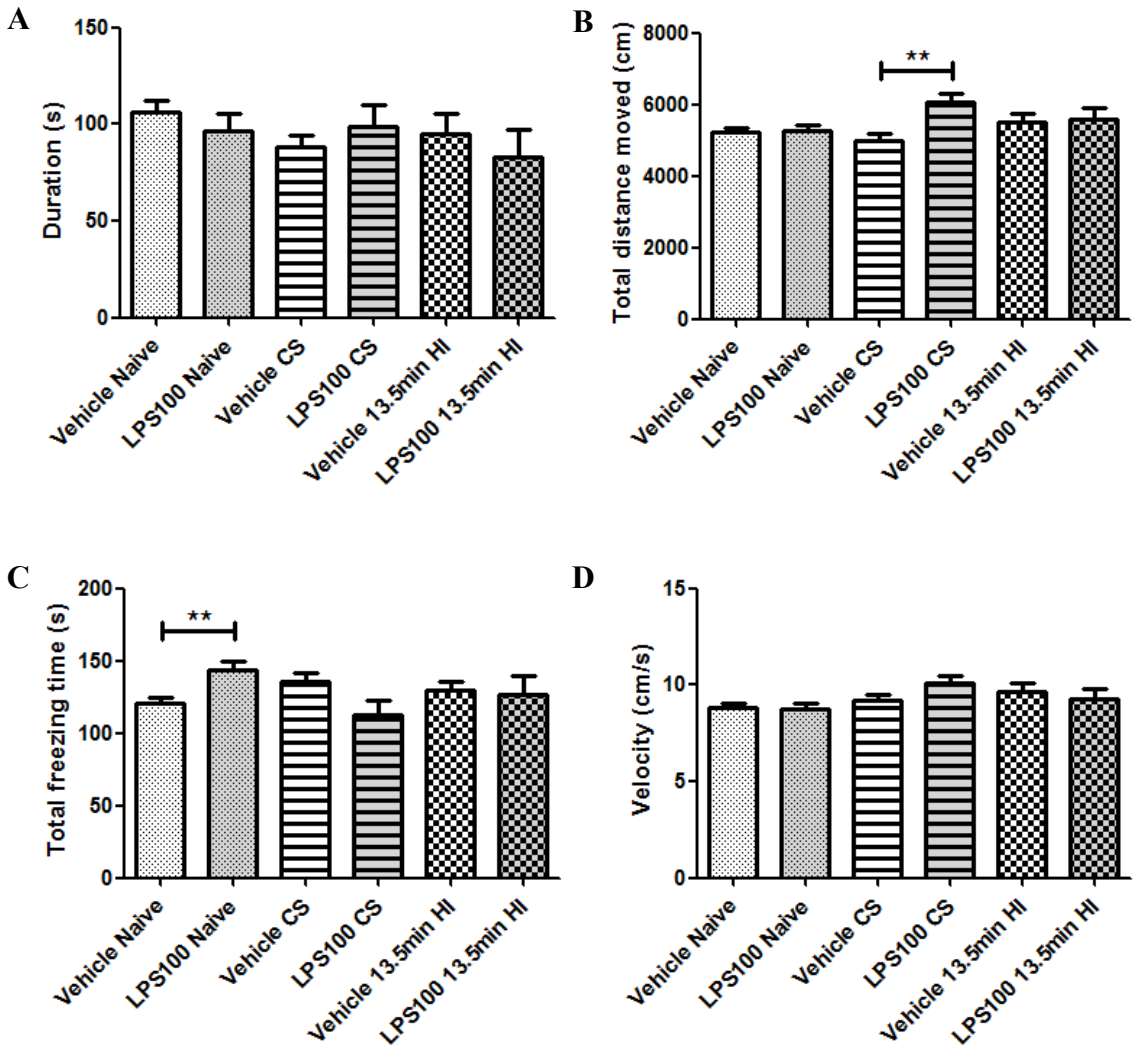
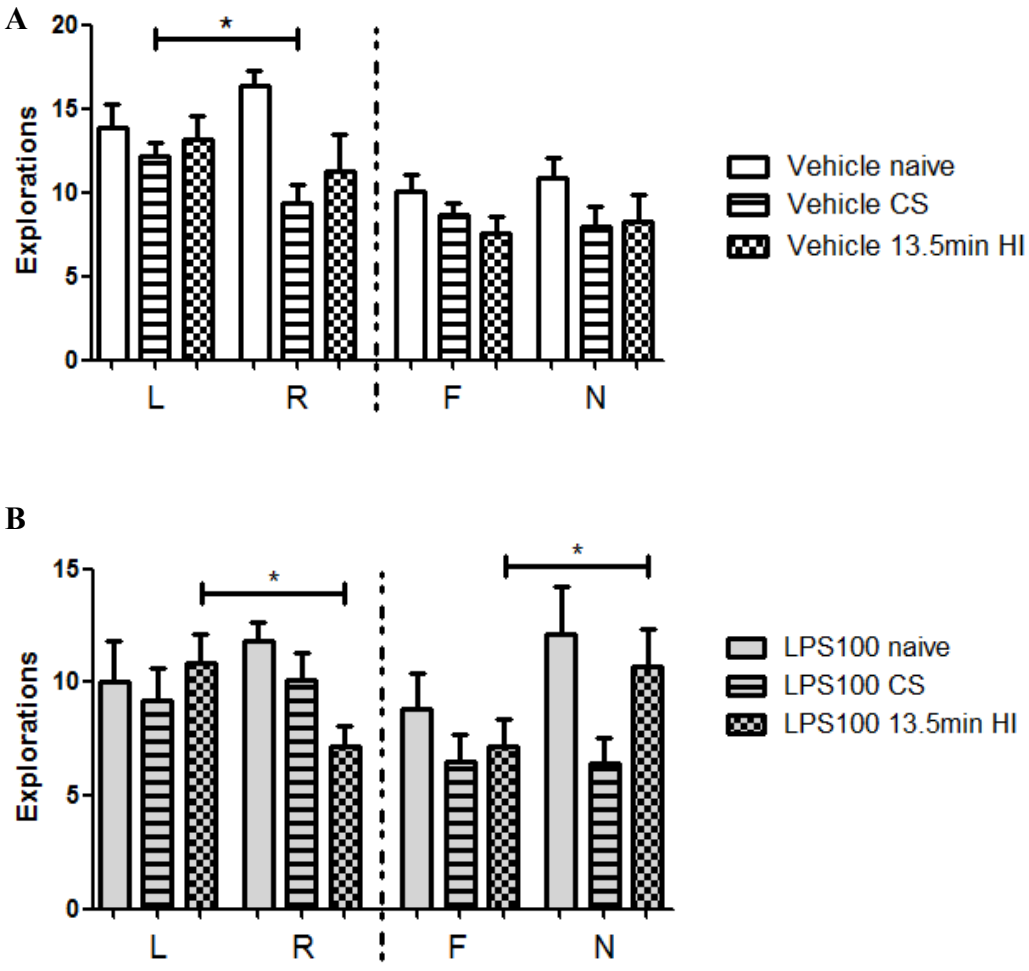
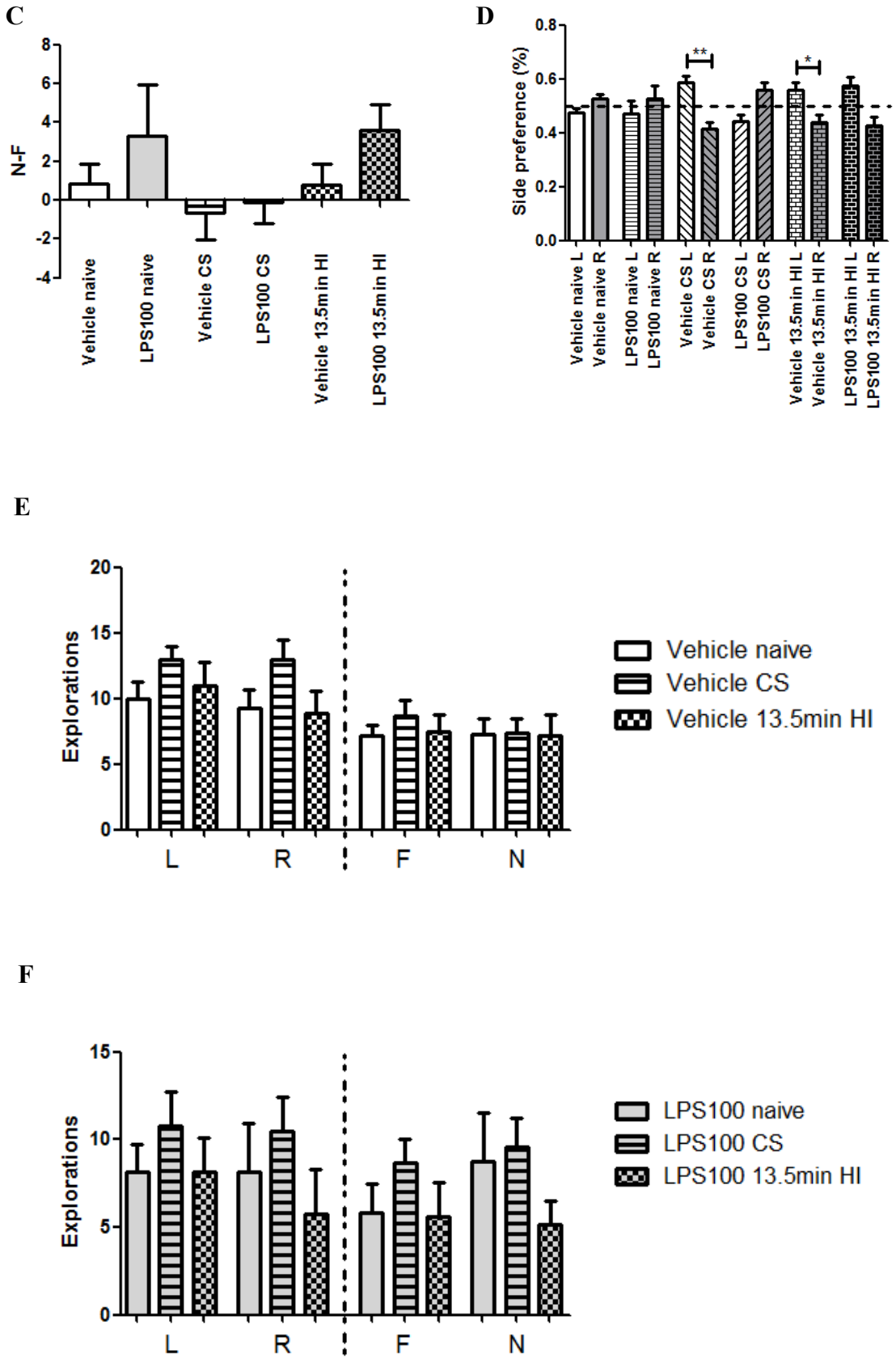


Figure 4.11 Open field tests of animals which were subjected to systemic maternal inflammation at P45. **A.** The duration (second) of animals spent in the centre zone during whole 10 minutes test. **B**. Total distance the animals moved during test. **C.** Total freezing time of the animals during the test. **D.** The average velocity of animals during the test. When comparing the effects of LPS injection on same experiment groups, data were analysed using student t-test. When comparing the effects of hypoxic-ischaemia with or without low dose LPS maternal inflammation, data were analysed using one-way ANOVA test with Bonferroni post test selected if the data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. Vehicle: naïve: n=18; CS: n=24; 13.5min H: n=13; LPS100: naïve: n=7; CS: n=10; 13.5min HI: n=7. *p<0.05, **p<0.01.

4.5.8.2 NOR tasks

At P45, only animals in the LPS100 + 13.5min HI group showed more interest in the novel object than the familiar object (Figs 4.12 A and B). However, there was no statistically significant difference in delta values (explorations to novel object minus familiar object) between the groups (Fig 4.12 C). It is noticed that animals in the vehicle CS and vehicle 13.5min HI groups had a preference to approach to the object on the left side (Fig 4.12 D). At P90, none of the experimental group had shown interest in an object which was relatively new in the P90 test or on either side physically (Figs 4.12 E-H). Among all the groups, the vehicle naïve group was the only group that had significantly more visits to the objects at P45 than at P90 (Fig 4.12 I).

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Vehicle 13.5min HI
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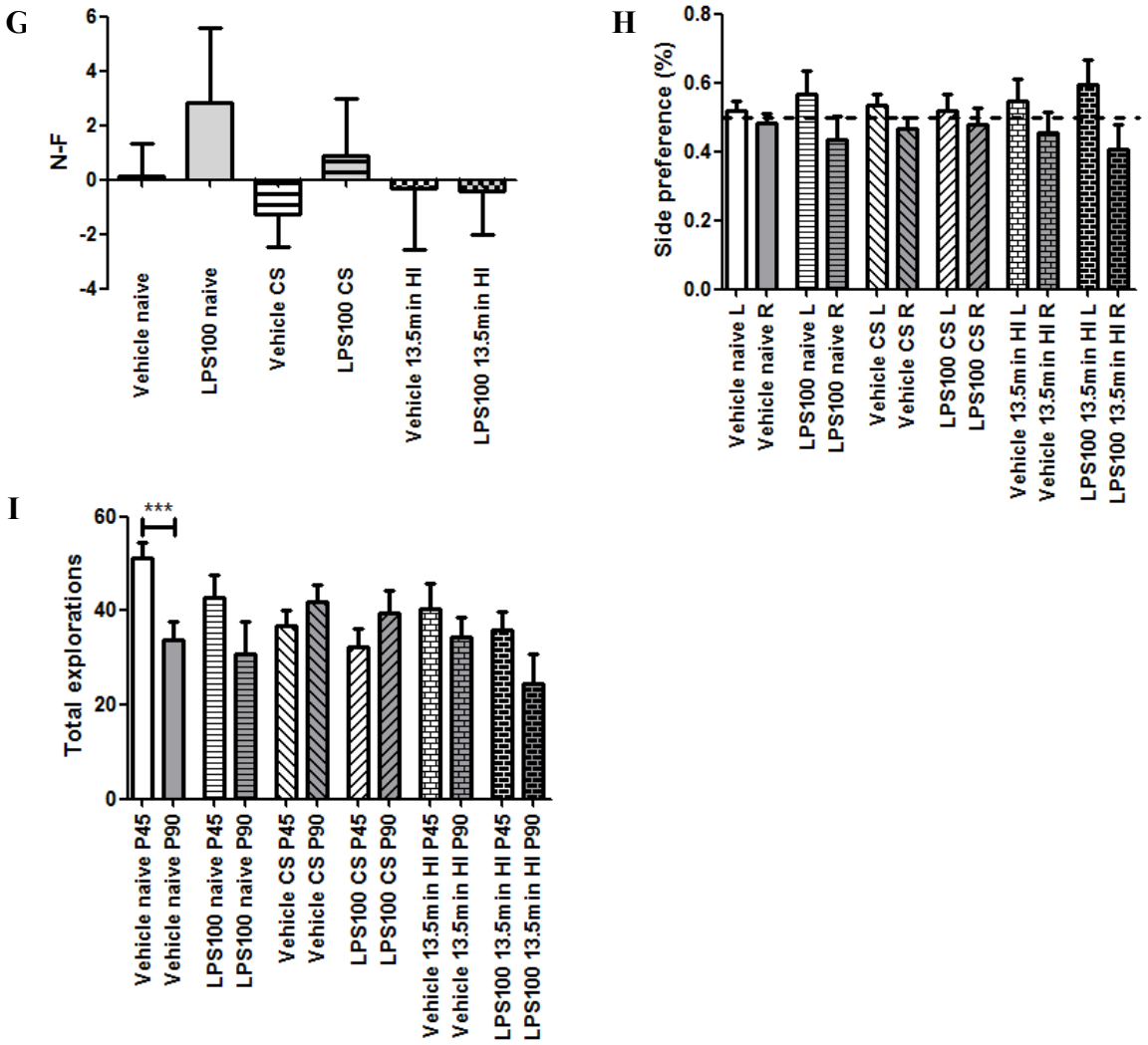


Figure 4.12 NOR test performance of different groups of animals at P45 and P90. A, B and E, F. The number of explorations of each object in vehicle groups (**A**), LPS100 groups (**B**) at P45 and vehicle groups (**E**), LPS100 groups (**F**) at P90. Data were analysed using Student t-test. If the data were parametric, paired t-test was used; if data were non-parametric, Wilcoxon matched pairs test was used. **C and G**. The delta values (explorations to novel object minus familiar object) of different groups of animals at P45 (**C**) and P90 (**G**). When comparing data between animals in vehicle or LPS100 groups, data were analysed using one-way ANOVA test with Bonferroni post test if data were parametric and Kruskal-Wallis test and Dunns post test if data were nonparametric. When comparing animals in the same surgical groups between vehicle and LPS100 groups, data were analysed using Student t-test. **D and H.** Side preference during the NOR test at P45 (**D**) and P90 (**H**). Data were analysed using Student t-test. If the data were parametric, paired t-test was used; if data were non-parametric, Wilcoxon matched pairs test was used. **I.** Total explorations during NOR tests. Data were analysed using Student t-test. If the data were parametric, paired t-test was used; if data were non-parametric, Wilcoxon matched pairs test was used. Vehicle groups: naïve, n=18; CS, n=24; 13.5min HI, n=13; LPS100 groups: naïve, n=7; CS, n=10; 13.5min HI, n=7. Data are expressed as mean ± SEM *p<0.05, **p<0.01, ***p<0.001

4.5.9 H&E staining at P90

After behavioural tests at P90, brain damage was examined using H&E staining. There were no lesions or dead neurons found in any of the groups (data not shown).

4.6 Discussion

It has been suggested that maternal infection exacerbates HI brain damage. Different studies have described animal models to mimic this situation and showed various results. Some claimed that a single dose LPS administration can protect the neonatal brains [215, 216] while the others demonstrated the opposite effect [210, 212, 217]. This study investigates this relationship by combining LPS induced inflammation with a new mouse model of HIE.

In this study, the systemic inflammation was induced using tail vein i.v. injection instead of i.p. injection. Firstly, i.p. injected LPS tends to cause relatively localised inflammation compared to i.v., therefore it was deemed more appropriate to use i.v. injection when a systemic inflammation is to be studied. Secondly, in this study mice were near the end of their pregnancy; therefore, in order to give an i.p. injection, gas anaesthesia would have to be used prior to the procedure. The strain, dosage and timing of LPS were picked based on other studies using rat model [218, 219]. This is the first time that a low dose LPS-induced systemic maternal inflammation and perinatal asphyxia mouse model were combined in an effort to try and understand the effect of a double-insult on the neonatal brain.

In Larouche's study, 200 μg/kg i.p. injections were administered every 12 hours starting from E17 on the rat and that led to the lowest maternal, fetal and early neonatal mortality observed [219]. In the present study, no maternal mortality was observed in any experimental group. The body weight changes from E14 – E18 and the stillbirth rates indicate that the i.v. LPS

was able to successfully induce systemic inflammation and that an increase of fetal mortality occurred when a higher dosage of LPS was used. Also, as expected, most of the inflammation-related factors increased in all 4 tissues examined after administration of LPS. These up-regulated changes of inflammatory factors confirmed that the LPS i.v. injection had successfully induced a systemic inflammation in the dams. IL-4 and IL-10 are not increased, which is not surprising, as these cytokines are classed as anti-inflammatory. In this study, the dose of 100 μg/kg provided similar late pregnancy outcomes and the 1 hour survival rate of the pups after 15 minutes of HI when compared to saline administration. It was frustrating and time consuming to have to re-establish the HIE model as none of the pups survived after 15min HI in the new location. Consequently, after comparing the survival rates of a range of HI times, 13.5min HI was chosen as it provided similar results to previous experiments. The LPS100+13.5min HI showed no difference in pup survival rate with the vehicle+13.5min HI group, and intriguingly, they both were significant higher than the saline+13.5min HI although this could be due to the small numbers of animals in the saline group.

Hypoxia inducible factor-1α (HIF-1α) is a transcription factor that responds to changes such as decreasing oxygen concentration in the cellular environment. Studies using neonatal stroke animal models have demonstrated the HIF-1α protein levels peaked at 3 hours [220] and 8 hours [221] from the initial hypoxic insult. However, the durability of HIF-1α protein and gene expression (8-24 hours after ischaemia) has been noted in global ischemia [222, 223]. An *in vitro* study has shown that HIF-1α protein levels increased remarkably in a macrophage-derived cell line after 1 μg/mL LPS treatment and peaked at 6-8 hours [224]. In the present study, the HIF-1α almost had no expression in the pup brain of P1 LPS100+HI13.5 animals, which was significantly lower than both the vehicle+HI13.5 and LPS100+naïve group. This result indicates that those pups survived after the double-hit

caused by LPS induced maternal inflammation and HI and that they may a have strong tolerance to adverse conditions.

As mentioned previously IL-1β plays an important role after HI damage. Nevertheless, IL-1β is one of the main cytokines that is involved in the inflammatory process induced by LPS. One study that combined LPS induced maternal inflammation and HI brain damage has shown that IL-1β protein levels increase in the pup brain 30 minutes after HI [218]. However, as mentioned in chapter 3, the IL-1β mRNA peak showed a decrease after 1 hour post HI damage in pup brain. Thus, in this study, P1 and P3 were chosen for measuring the protein levels of IL-1β. An increase in IL-1β levels was seen in the maternal tissues after LPS injection, which may indicate an up-regulation of IL-1β expression in the pup brains after dams were injected with LPS. However, the only significant difference observed in this study was in the naïve P1 groups between vehicle and LPS treatment and that indicated LPS administration had an effect on naïve pups. It is intriguing to find that there was no significant difference of IL-1β expression between the vehicle and LPS administration groups in CS or HI group.

Studies using human choriodecidua explants and chorioncarcinoma cell lines have shown that p38 MAPK activation occurs in response to LPS [225, 226]. It would be interesting to identify how the fetal brain responds to LPS-induced maternal inflammation. However, due to time limitation it was only the double-insult towards the postnatal pup brain that this study was able to focus on. The data regarding to phospho-p38 showed remarkable increase in LPS treated CS and HI groups compared with vehicle groups at P1. However, the phosphorylation of p38 can also be a result of apoptosis [227-229]. Apoptotic cell death plays an important role in neurodegeneration after HI brain injury [163]. Activated caspase-3, Bcl-2 family of proteins and STAT-3 play important roles in neuronal apoptosis after HI [55, 202, 203, 230]. A recent study showed that low doses of repeated LPS administration to fetal sheep provided

neuroprotection in the developing brain by attenuating apoptosis [231]. In this study, other than the changes in phospho-p38 levels mentioned above, only phospho-bad protein level was found to be significantly increased and decreased in LPS100+HI13.5 than LPS100+CS groups at P1 and P3 respectively. These data suggest that LPS administration during late pregnancy plus HI combination may enhance apoptosis in pup brain.

This study aims to investigate the consequence of a low dose LPS induced systemic inflammation during late pregnancy and since long-term neuronal deficiency is an important aspect of HI damage, hence several behavioural tests were performed. OFT has been widely used to test general locomotor activity levels and anxiety in rodents in scientific research and willingness to explore in rodents. In the present study, no motor deficit was observed in the OFT test. However, it is noted that the naïve animals which had LPS100 had higher levels of anxiety than the vehicle naïve animals. The NOR tasks have become a widely used model for investigation into memory alterations. In this study, it is very interesting to find that only mice from the LPS100+HI13.5 group spent more time in exploring the novel objects at P45 which was when the first time the test was performed. By comparing the total explorations performed at P45 and P90, it has been found that only vehicle naïve animals had less motivation to explore the objects at P90, which indicates that all other groups had worse long-term memory when compared to the vehicle naïve animals.

In summary, these data suggest that 4 days continuous LPS administration in late pregnancy in mice led to increased fetal mortality and apoptosis in postnatal pup brains, but did not worsen the long-term outcomes of those pups when further exposed to HI. However, the number of control animals needs to be increased in order to draw a more convincing conclusion. This study would benefit from the addition of a saline i.v. control group but because of time restraints this will have to be performed later.

Chapter 5. The effects of DHA maternal supplementation on the HIE mouse model

5.1 Introduction

Long-chain polyunsaturated fatty acids are a major component of brain membrane phospholipids. In particular, docosahexaenoic acid (DHA) is highly enriched in brain, nervous tissue and retina [89, 90]. Docosahexaenoic acid is an omega-3 polyunsaturated fatty acid (n-3 PUFA) which can be found in cold-water oceanic fish oils and microalgae. DHA can also be obtained by synthesis from alpha-linolenic acid (ALA) which is also an n-3 PUFA found in seeds, nuts and many vegetable oils. DHA accumulates in the brain of neonates during the late prenatal stage which corresponds to the third trimester of pregnancy in humans and the last 3 days of gestation and also during the early postnatal period in rats [104, 232]. This accumulation depends on the amount and types of dietary intake of n-3 fatty acids and n-6 fatty acids which interact and compete with n-3 fatty acids in the fatty acid metabolic pathway [97, 98, 233]. Thus, it is crucial that pregnant women consume a diet which contains n-3 PUFAs.

An inadequate supply of DHA through several generates has been found to cause memory deficiency, learning difficulties and visual acuity loss on offspring animals [234, 235]. Cohort and laboratory studies have shown that oily fish intake reduces the incidence of stroke [115, 120, 236-239]. However, the neuroprotective mechanisms behind these effects are not fully understood, some have suggested that n-3 PUFAs especially DHA have the anti-inflammatory effects which protects the brain from HI injury [115, 116]. These anti-inflammatory processes may be involved with changes in membrane fluidity and some cell signalling pathways, such as toll-like receptor-4 (TLR-4) [238, 240, 241]. Studies have also shown that another key anti-inflammatory effect is modulating the levels of inflammatory related factors such as NF-κB and IL-1β [115, 242]. Another possible neuroprotective mechanism of DHA is the anti-apoptosis effect. One *in vitro* study has shown that DHA and its mediator neuroprotectin D1 (NDP1) can up-regulate Bcl-2 family anti-apoptotic proteins

including Bcl-2 and Bcl-xL, but down-regulate pro-apoptotic proteins including Bax and Bad and reduce caspase-3 activation [243]. Another study using a rat cerebral ischaemia model has shown that DHA intraperitoneal administration before ischaemic insult increased Bcl-2 gene expression as well as decreased active caspase-3 protein level [117]. Moreover, in addition to short term cellular changes, other studies using different animal models have claimed that DHA could improve cognitive functioning [244, 245].

5.2 Aims

1) To define the amount of fatty acids contents in different experiment diets.

2) To investigate the amount of fatty acids contents in the maternal blood and P1 pup brain after 16-days of specific diet.

3) To determine if and how DHA enriched diet improves the outcome of neonates who suffered HIE brain damage.

5.3 Hypothesis

Giving a DHA enriched diet to dams could improve both short-term and long-term outcomes of neonates who suffered HIE brain damage.

5.4 Materials and Methods

All diets were made by Special Diets Services (SDS), UK. Rat and Mouse No.1 maintenance autoclavable diet (RM1) was used when the HIE model was established, as supplied by the animal facilities at the university. Subsequently on gestational day 2 (E2) mice were randomly assigned into two experimental groups: a control group fed a control diet or a DHA enriched group fed on the control diet supplemented with 1.5% DHA as measured in relation to the total fat content of the diet. The total fatty acids content for the RM1 and the customised diets (control and DHA diets) according to the manufacturer datasheet were: 2.27% and 10.2% respectively. The same diets were fed to the dams and pups after birth.

On E18, HIE was induced as previously described in chapter 2. As previously mentioned, pups exposed to 15 minutes hypoxia had a worse outcome than the 10 minutes hypoxia, explaining why 15 minutes was chosen as the time of asphyxia in this study. Maternal red blood cells (RBCs) and plasma were collected for fatty acids analysis after terminal anaesthesia by cardiac puncture on the morning of E18, before the initiation of labour in a

cohort of dams not used for perinatal hypoxia induction. Pup brains samples were collected at P1 and P3.

The total fatty acids of samples were extracted by the method of Folch *et al* [157]. The volumes of different samples used in the fatty acid analysis were: 500mg of diet, 500µl of maternal plasma, 250µl of RBC and P1 whole pup brains. The resulting fatty acid methyl esters (FAMEs) were separated by gas chromatography which was carried by Mr Yiqun Wang.

Gas chromatography (GC) is used commonly in analytical chemistry for separating and analysing compounds that can be vaporized without decomposition. It has been widely used for lipid analysis [246]. Briefly, the stationary phase (in this study, the FAMEs) is carried by a carrier gas, nitrogen, through a narrow tube and are identified electronically upon exit. The various analytes are separated as they progress through the column and reach the end of the column at different times and therefore have varying retention times. A chromatogram was then generated and by calculating the area of the peak of the chromatogram, the concentration of an analyte in the original sample can be determined.

To determine protein levels of apoptosis, hypoxia and inflammatory related markers, P1 and P3 pup brain samples were analysed using multiplex techniques which has been previously described in chapter 2.

5.5 Results

5.5.1 Survival rates of HIE model and short term behavioural changes after perinatal HI

Pup survival rates at 1 hour are shown in Fig 5.1. The CS and 15 min HIE groups of control diet were 95.5% and 31% while the survival rates for CS and 15 min HIE groups of DHA diet were 98.4% and 37.9%. Data were analysed using chi-square by a 2x2 contingency table. The association between the control and DHA enhanced diets and pup survival rates after having HIE was considered to be not statistically significant (Table 5.1). However, when comparing the data of both customised diets separately to the RM1 diet (the lowest total fatty acid content of the 3 diets) the association between the two diets and survival rates after 15 minutes HIE was statistically significant (Tables 5.2 and 5.3).

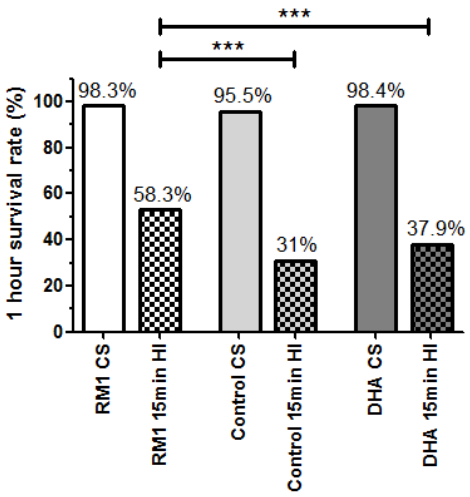


Figure 5.1. One hour pup survival rates of 3 different experimental diets. Data were analysed using chi-square with Yates correction by a 2x2 contingency table. RM1 diet: CS (n=289, 59 surgeries), 15min HI (n=416, 59 surgeries); control diet: CS (n=111, 24 surgeries), 15min HI (n=178, 25 surgeries); DHA enhanced diet: CS (n=127, 29 surgeries, 15min HI (n=190, 30 surgeries).

Table 5.1 Survival analysis for animals after 15min HI fed with control and DHA enhanced maternal diets

15min HI	Mortality	Survival	Total
Control diet	123	55	178
DHA enhanced diet	118	72	190
Total	241	127	368

Chi squared equals 1.693 with 1 degree of freedom. The two-tailed P value equals 0.1933. The association between rows and columns is considered to be not statistically significant.

Table 5.2 Survival analysis for animals after 15min HI fed with control and RM1 maternal diets

15min HI	Mortality	Survival	Total
Control diet	123	55	178
RM1 diet	194	222	416
Total	317	277	594

Chi squared equals 24.388 with 1 degree of freedom. The two-tailed P value equals 0.0001. The association between rows and columns is considered to be extremely statistically significant.

Table 5.3 Survival analysis for animals after 15min HI fed with DHA enhanced and RM1 maternal diets

15min HI	Mortality	Survival	Total
DHA enhanced diet	118	72	190
RM1 diet	194	222	416
Total	312	294	606

Chi squared equals 11.886 with 1 degree of freedom. The two-tailed P value equals 0.0006. The association between rows and columns is considered to be extremely statistically significant.

5.5.2 Fatty acid analysis in diets, dams and neonates

To determine whether the DHA enriched diet could increase the level of DHA in maternal tissue and pup brains, fatty acid contents in experimental diets (Table 5.4 and Fig 5.2), maternal RBC and plasma (Table 5.5 and Fig 5.3) and pup brains (Table 5.6 and Fig 5.3) had been evaluated. Values are expressed as percentage of total fatty acids. The amount of linoleic acid (LA) and alpha-linolenic acid (ALA) were significantly higher in RM1 diet than the other 2 experiment diets and there was no difference between control and DHA diets (Figs. 5.2 A and B). RM1 diet and control diet contained no DHA while as the DHA diet contained 0.8% of DHA in total amount of fatty acids (Fig 5.2 C). Arachidonic (AA) and eicosapentaenoic acids (EPA) were not detected in all 3 diets (data not shown). Five PUFAs (LA, ALA, AA, EPA and DHA) levels in the maternal plasma, maternal RBC and pup brain samples after consuming RM1, control and DHA enhanced diets are shown in Fig 5.3. In maternal plasma, there was significantly higher amount of LA after being fed with both RM1 diet and the DHA enhanced diet than the control diet (Fig 5.3 A). After being fed with RM1, the maternal RBC contained higher LA than the other two groups (Fig. 5.3 A). In P1 pup brain, the concentration of LA was significant higher after being fed with RM1 diet than the control diet and there is no significant difference between control and DHA diets (Fig 5.3 A). Statistically higher amounts of ALA in the maternal plasma and maternal RBC were found after mice were fed with RM1 diet (Fig. 5.3 B). No statistical difference was found between groups in pup brain (Fig 5.3 B). With respect to AA, a significant increase was observed in maternal plasma after being fed with control diet than both RM1 and DHA enhanced diets while in maternal RBC the trend was opposite (Fig. 5.3 C). In pup brain, the amount of AA in both RM1 and control diet groups were at a similar level and were both statistically higher than the DHA enhanced diet group (Fig 5.3 C). EPA in maternal blood were significantly higher in the RM1 and DHA diet groups than the control group while as in pup brain the

amount of EPA was higher in the DHA group (Fig 5.3 D). Significantly higher DHA was found in maternal plasma after being fed with DHA enhanced diet than the other two experimental diet groups (Fig. 5.3 E). In maternal RBC and pup brain data, the concentration of DHA was significantly lower in the control group than the other two diet groups (Fig 5.3 E).

Table 5.4 Fatty acid contents in three experimental diet groups

	Diets		
	RM1	**Control**	**DHA**
c16:0	16.65 ± 0.23	13.17 ± 0.04	13.26 ± 0.03
c16:1n-7	0.12 ± 0.00	0.09 ± 0.00	0.14 ± 0.00
c18:0	1.79 ± 0.01	8.97 ± 0.05	8.94 ± 0.03
c18:1n-9	15.29 ± 0.05	49.56 ± 0.24	48.7 ± 0.10
c18:1n-7	1.28 ± 0.03	1.26 ± 0.05	1.28 ± 0.04
c18:2n-6 (LA)	**57.89 ± 0.16**	**5.07 ± 0.03**	**4.98 ± 0.02**
c18:3n-3 (ALA)	**5.41 ± 0.04**	**0.08 ± 0.00**	**0.07 ± 0.00**
c20:0	0.11 ± 0.01	0.17 ± 0.01	0.15 ± 0.03
c20:1	0.41 ± 0.03	0.12 ± 0.00	0.12 ± 0.00
c22:0	0.09 ± 0.02	0.23 ± 0.02	0.25 ± 0.02
c22:6n-3 (DHA)	**0 ± 0.00**	**0 ± 0.00**	**0.78 ± 0.04**

Value expressed as percentage of total fatty acids. Data are expressed as mean ± SEM. n=6 in each group. Highlighted data were used to generate Fig 5.2.

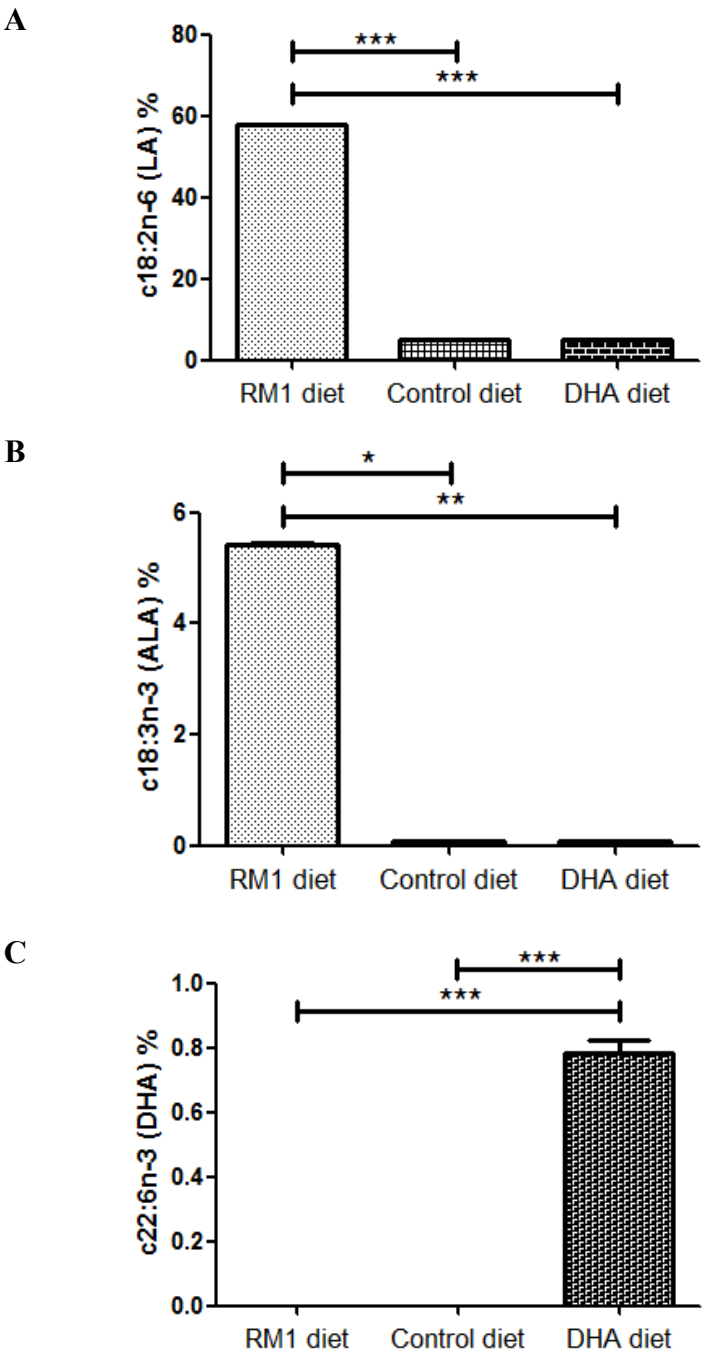


Figure 5.2. Fatty acid contents of 3 different experimental diets. Data in these figures were generated using those highlighted in Table 5.4. Three different long-chain fatty acids, LA (**A**), ALA (**B**) and DHA (**C**), levels in the experimental diets were measured using Folch method. Data were analysed using one-way ANOVA test with Bonferroni post test if the data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. n=6 in each group; *p<0.05, **p<0.01, *** p<0.001.

Table 5.5 Fatty acid contents in the maternal plasma and RBC samples of three experimental diet groups

	Maternal Plasma			Maternal RBC		
	RM1	**Control**	**DHA**	**RM1**	**Control**	**DHA**
c16:0	25.47±0.38	21.72±0.25	19.19±1.16	30.55±0.23	26.17±0.17	25.25±0.73
c16:1n-7	2.69±0.27	2.73±0.23	1.53±0.24	0.55±0.09	0.71±0.04	0.67±0.10
c18:0	8.96±0.51	10.07±0.58	12.34±1.23	15.21±0.27	13.39±0.22	12.71±0.35
c18:1n-9	17.01±0.77	34.18±0.69	31.16±1.24	10.30±0.29	20.10±0.45	21.31±0.59
c18:1n-7	1.04±0.09	1.28±0.10	2.56±0.29	0.81±0.04	0.95±0.10	2.24±0.07
c18:2n-6 (LA)	**24.28±0.93**	**7.23±0.15**	**11.81±1.15**	**8.57±0.28**	**4.40±0.07**	**6.90±0.48**
c18:3n-3 (ALA)	**0.96±0.08**	**0.03±0.01**	**0.04±0.01**	**0.13±0.02**	**0.01±0.00**	**0.01±0.00**
c20:0	0.09±0.01	0.09±0.01	0.11±0.01	0.19±0.02	0.14±0.02	0.15±0.01
c20:1	0.17±0.02	0.32±0.02	0.41±0.05	0.19±0.01	0.36±0.02	0.38±0.03
c20:3n-9	0.18±0.02	0.07±0.02	0.06±0.00	0.21±0.03	0.12±0.01	0.03±0.00
c20:3n-6	0.36±0.02	0.36±0.03	0.98±0.20	0.71±0.02	0.71±0.02	1.13±0.04
c20:4n-6 (AA)	**9.48±0.88**	**9.55±0.49**	**8.34±0.75**	**17.22±0.23**	**17.81±0.49**	**14.93±0.50**
c20:3n-3	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.03±0.00	0.02±0.00
c22:0	0.23±0.03	0.34±0.03	0.27±0.02	0.78±0.02	0.69±0.02	0.51±0.03
c22:1n-9	0.02±0.00	0.03±0.01	0.01±0.00	0.04±0.01	0.04±0.01	0.01±0.00
c20:5n-3 (EPA)	**0.29±0.03**	**0.05±0.01**	**0.34±0.09**	**0.27±0.01**	**0.12±0.00**	**0.27±0.03**
c22:2n-6	0.06±0.01	0.08±0.01	0.09±0.01	0.12±0.01	0.09±0.01	0.09±0.01
c22:4n-6	0.43±0.04	0.15±0.01	0.06±0.01	1.85±0.06	1.55±0.02	0.67±0.02
c24:0	0.26±0.03	0.35±0.03	0.42±0.03	1.17±0.01	1.02±0.03	1.01±0.03
c22:5n-6	0.72±0.09	1.39±0.12	0.28±0.06	1.09±0.07	1.97±0.08	0.58±0.03
c24:1	0.07±0.01	0.06±0.00	0.20±0.03	0.32±0.01	0.64±0.01	0.83±0.04
c22:5n-3	0.73±0.03	0.01±0.01	0.05±0.01	0.76±0.04	0.10±0.00	0.18±0.01
c22:6n-3 (DHA)	**2.31±0.19**	**0.83±0.03**	**4.38±0.21**	**4.64±0.11**	**2.87±0.08**	**7.07±0.18**

Value expressed as percentage of total fatty acids. Data are expressed as means ± SEM. n=6 in each group except for DHA RBC's where n=4. Highlighted data were used to generate Fig 5.3.

Table 5.6 Fatty acid contents in the pup brain samples of the three experimental diet groups

	Pup brain		
	RM1	**Control**	**DHA**
c16:0	32.21±0.04	31.46±0.17	31.07±0.07
c16:1n-7	2.61±0.29	2.67±0.09	2.49±0.03
c18:0	17.53±0.10	16.72±0.09	16.67±0.07
c18:1n-9	12.10±0.08	14.77±0.17	13.79±0.21
c18:1n-7	1.82±0.04	2.00±0.05	3.58±0.07
c18:2n-6 (LA)	**0.89±0.00**	**0.48±0.02**	**0.59±0.02**
c18:3n-3 (ALA)	**0.01±0.01**	**0.00±0.00**	**0.00±0.00**
c20:0	0.06±0.01	0.05±0.01	0.07±0.00
c20:1	0.18±0.00	0.28±0.01	0.30±0.01
c20:3n-9	0.08±0.02	0.05±0.02	0.04±0.00
c20:3n-6	0.36±0.17	0.26±0.02	0.39±0.03
c20:4n-6 (AA)	**11.72±0.00**	**11.55±0.14**	**10.29±0.06**
c20:3n-3	0.01±0.02	0.02±0.01	0.02±0.00
c22:0	0.21±0.01	0.19±0.01	0.15±0.00
c22:1n-9	0.03±0.02	0.01±0.01	0.01±0.00
c20:5n-3 (EPA)	**0.07±0.00**	**0.02±0.00**	**0.09±0.00**
c22:2n-6	0.05±0.08	0.04±0.00	0.05±0.00
c22:4n-6	2.61±0.01	2.44±0.07	1.16±0.02
c24:0	0.20±0.06	0.19±0.01	0.24±0.01
c22:5n-6	1.74±0.01	4.10±0.35	0.94±0.04
c24:1	0.04±0.01	0.04±0.00	0.06±0.01
c22:5n-3	0.30±0.50	0.01±0.01	0.17±0.01
c22:6n-3 (DHA)	**8.67±0.00**	**4.30±0.31**	**12.56±0.23**

Value expressed as percentage of total fatty acids. Data are expressed as means ± SEM. n=6 in each group. Highlighted data were used to generate Fig 5.3.

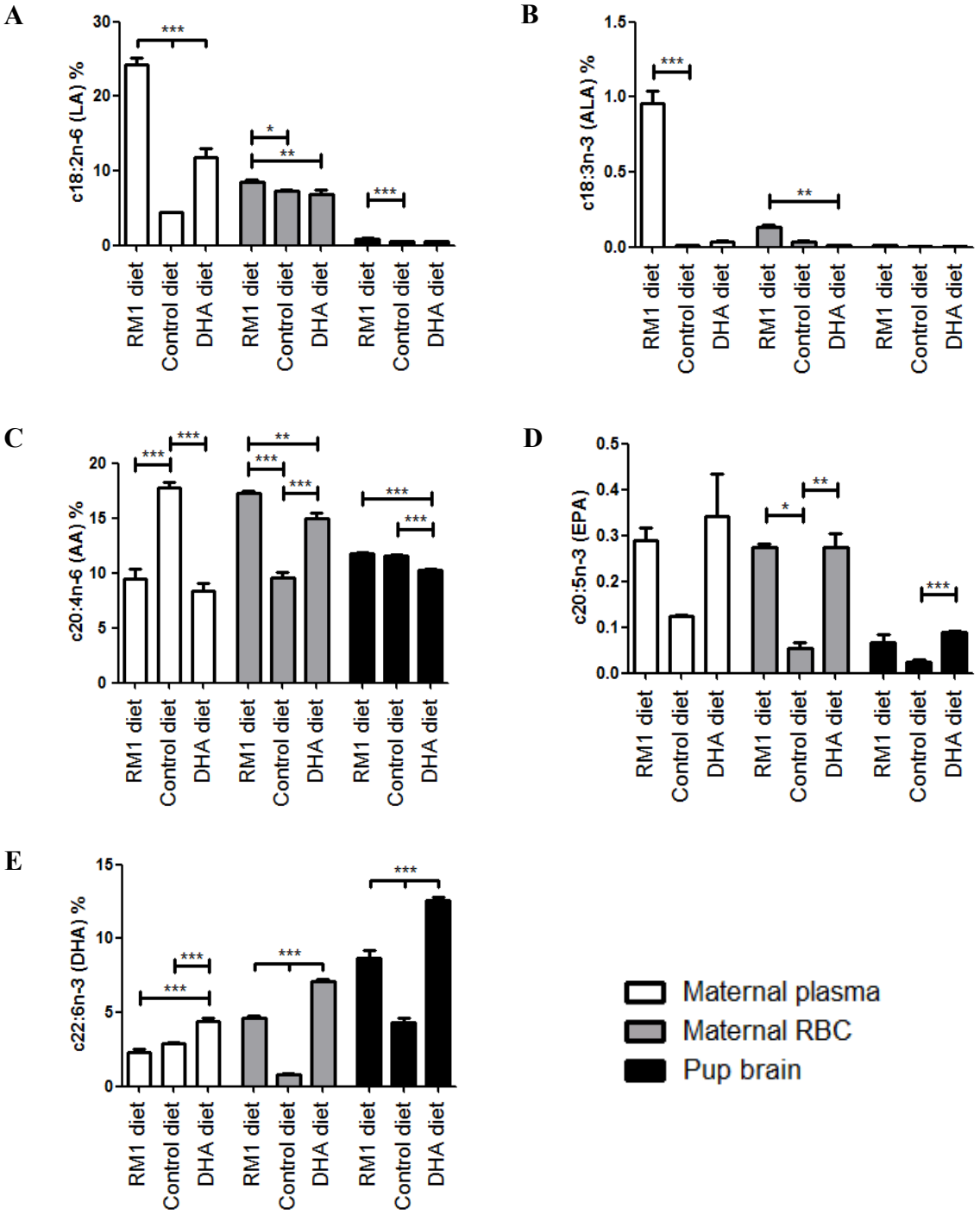


Figure 5.3. Fatty acid contents of maternal plasma, maternal red blood cell (RBC) and pup brain samples after feeding with 3 different experimental diets. Data in these figures were generated using those highlighted in Table 5.5 and Table 5.6. Five fatty acid levels were showed in the figures above, LA (**A**), ALA (**B**), AA (**C**), EPA (**D**) and DHA (**E**). All samples were collected at postnatal day 1 (P1) then processed using Folch method. Data were analysed using one-way ANOVA test with Bonferroni selected groups post test if data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. n=6 in each group except for DHA diet RBC's where n=4; *p<0.05, **p<0.01, *** p<0.0001.

5.5.3 Inflammation related IL-1β protein level expressed in pup brains

Previously in Chapter 3 (HIE model), the mRNA levels of inflammatory cytokines were measured and IL-1β showed increase after having HIE in pup brains. Therefore in this study, to determine whether the DHA enhanced maternal diet would cause an anti-inflammatory effect in the offspring, the inflammation related cytokine, IL-1β, was measured using a Milliplex assay. IL-1β was expressed similarly in both experimental diets group regardless of the degree of hypoxic ischaemia in the pups (Fig 5.4).

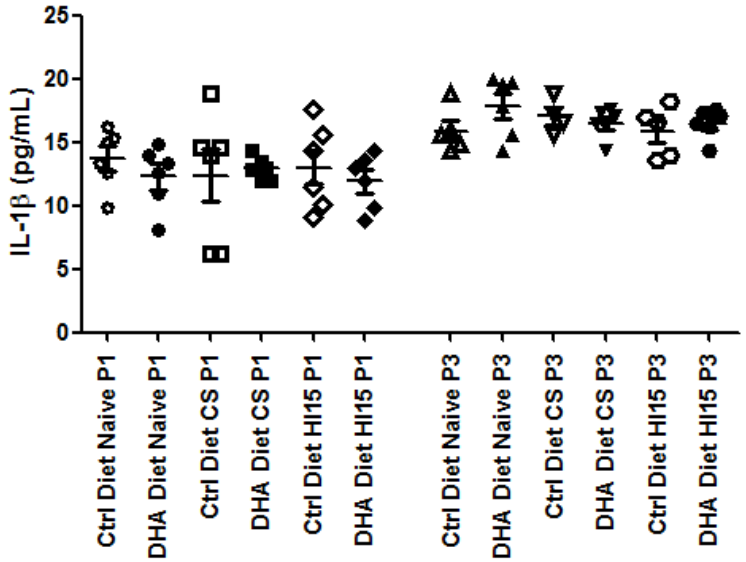


Figure 5.4. Inflammation related factor IL-1β protein level expressed in pup brains. Pup brains were collected at postnatal day 1 and 3. IL-1β protein level was measured using Multiplex assay. When comparing the effects of two different diets on same experiment group, data were analysed using student t-test. When comparing the effects of hypoxic-ischaemia on pup brains fed on one type of experiment diet, data were analysed using one-way ANOVA test with Bonferroni selected groups post test if the data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. n=5-6 in each group.

5.5.4 Hypoxic related protein level expression in pup brains

HIF-1α, a protein whose expression in tissues, reflects an environment with insufficient oxygen, was detected by BioPlex and the results for this protein are shown in Fig 5.5. When comparing the HIF-1α expression in the pup brains after two different customised maternal diets, significant differences were only found in the P3 15 minutes HI groups. Among the P1 pups in DHA enhanced diet groups, the CS group had a bigger increase in the HIF-1α expression than the naïve and 15 minutes HI group. However, this trend was not apparent in the P3 pups. Intriguingly, the HIF-1α levels on P3 were lower in the CS group and the DHA-enriched diet group.

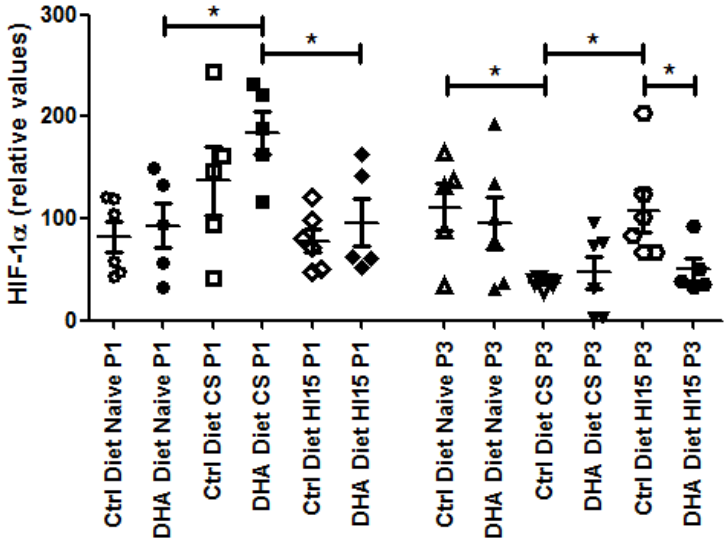


Figure 5.5. Hypoxia related factor HIF-1α protein level expressed in pup brains. Pup brains were collected at postnatal day 1 and 3. HIF-1α protein level was measured by MILLIPLEX. The fluorescence intensity (FI) value of positive control was 795.25.When comparing the effects of two different diets on same experiment group, data were analysed using student t-test. When comparing the effects of hypoxic-ischaemia on pup brains fed on one type of experiment diet, data were analysed using one-way ANOVA test with Bonferroni selected groups post test if the data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as mean ± SEM. n=5-6 in each group. *$p<0.05$, **$p<0.01$.

5.5.5 Apoptosis related proteins expressed in pup brains

Several apoptosis related proteins expressed in P1 and P3 pup brains were detected using BioPlex assays to determine whether the DHA enhanced diet modulated apoptosis. At P1, the levels of caspase-3 (Fig 5.6 A) and phospho-p38 (Fig 5.6 B) were significantly increased, for active caspase-3 expression, the levels were greater in the DHA naïve pup brains when compared with the control naïve, DHA CS and DHA HI groups. The control HI group had a significant increase expression of phospho-p38 than the control naïve and the DHA HI groups. At P3, There was no significant difference between groups in the levels of caspase-3, phospho-p38 and phospho-STAT3 expressions (Figs 5.6 A, B and C). However, the expression of phospho-bad in all the DHA enhanced groups were significantly higher than their matching control groups, but there was no significant difference among all the DHA groups as well as in the control group (Fig 5.6 D).

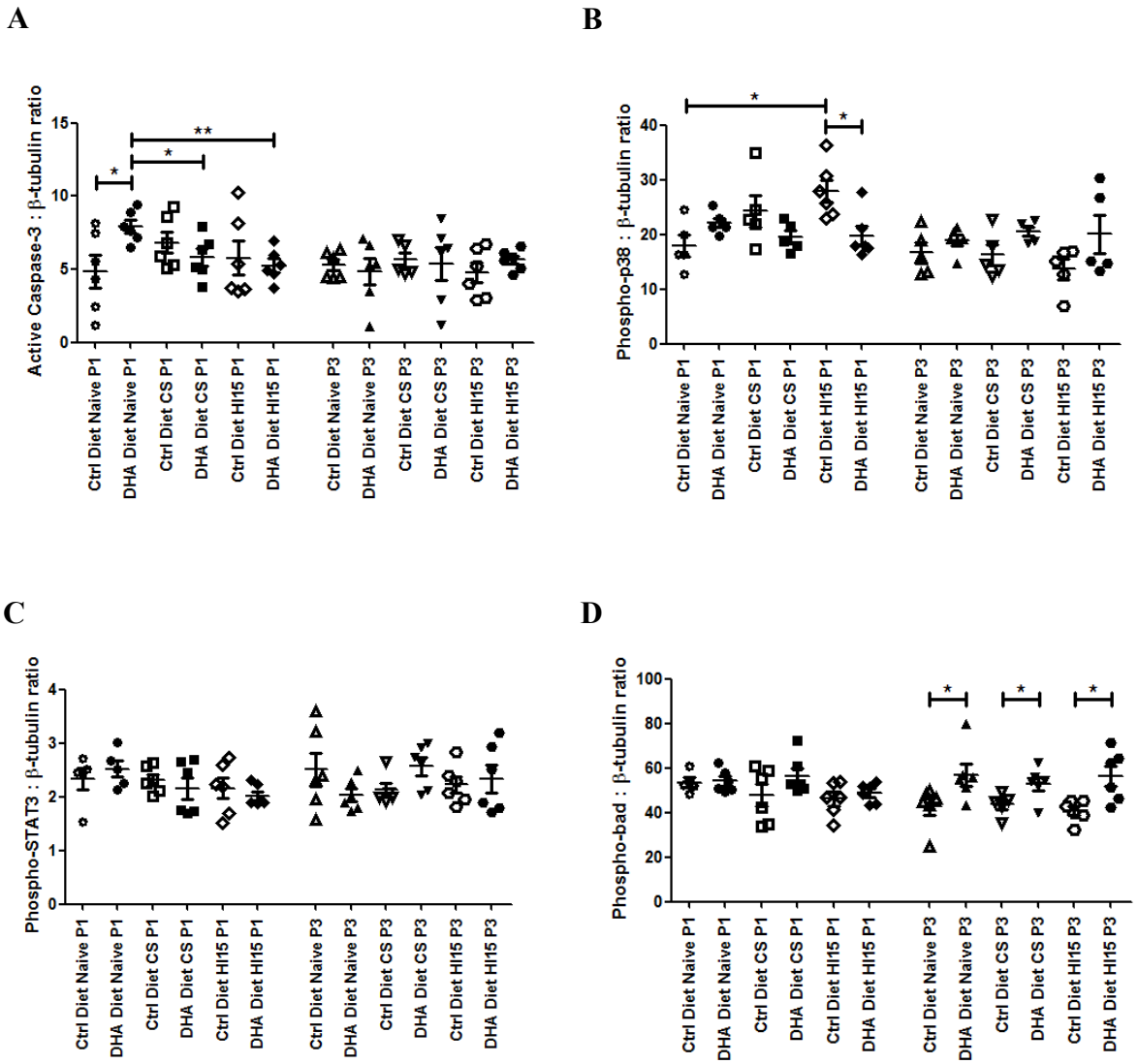


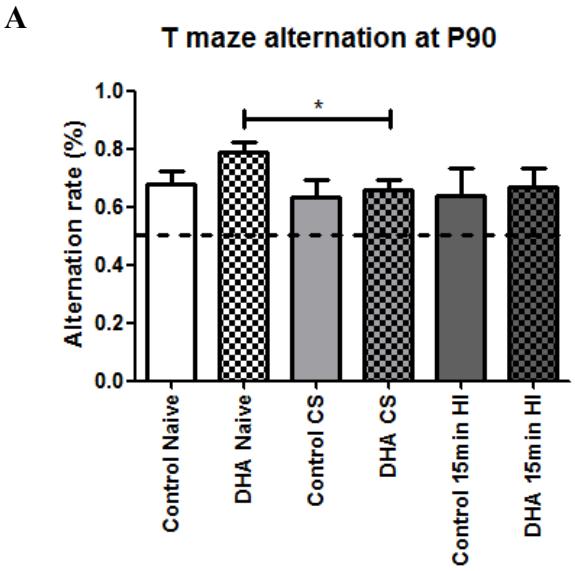
Figure 5.6. Apoptosis related factors protein levels expressed in pup brains. Pup brains were collected at postnatal day 1 and 3. Active Caspase-3 (**A**), Phospho-p38 (**B**), Phospho-STAT3 (**C**) and Phospho-bad (**D**) proteins levels were measured by MILLIPLEX. Data were normalised by β-tubulin. When comparing the effects of two different diets on same experiment group, data were analysed using student t-test. When comparing the effects of hypoxic-ischaemia on pup brains fed on one type of experiment diet, data were analysed using one-way ANOVA test with Bonferroni selected groups post test if the data were parametric and Kruskal-Wallis test and Dunns selected post test if data were nonparametric. Data are expressed as means ± SEM. n=5-6 in each group. *p<0.05, **p<0.01.

5.5.6 Long-term neurocognitive outcome

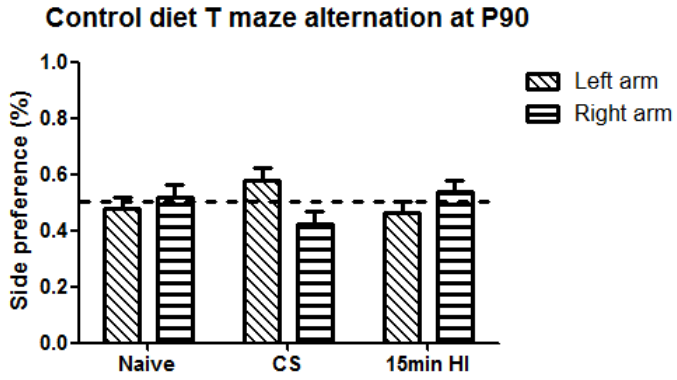
T-maze alternation test and novel object recognition test were used to determine if the maternal DHA enhanced diet could improve the long term neurocognitive outcome of the pups.

5.5.6.1 T-maze continuous alternation task

Working memory was assessed by performing a T-maze continuous alternation task. T-maze continuous alternation task was performed at P90 (Fig 5.7). There was no significant difference between groups when the animals were fed with control diet (Fig 5.7 A). The naïve group of animals who were fed with DHA enhanced diet had significant higher alternation rate than CS group (Fig.5.7 A). No significant difference between the same surgical groups of different experimental diets is seen (Fig.5.7 A). Also, there was no significant difference between groups in the side preference rate in both control and DHA groups (Figs.5.7 B and C).



B



C

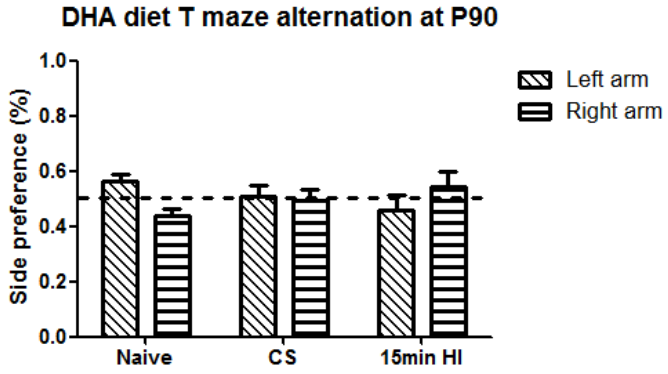


Figure 5.7. T-maze alternation of animals fed with control and DHA enhanced diets at P90. **A.** The alternation rate is calculated as number of alternations/total number of choices. The dotted line indicates 50%. Data were analysed using one-way ANOVA test with Bonferroni selected groups post test if data were parametric; and Kruskal-Wallis test and Dunns selected post test if the data were nonparametric. Student t-test was used when comparing data between control and DHA enhanced diets. **B and C.** Percentage of side preference. Student t-test was used. Data are expressed as mean ± SEM. Control diet: naïve, n=6; CS, n=4; HI 15min, n=5; DHA diet: naïve, n=5; CS, n=7; 15min HI, n=3. *$p<0.05$

5.5.6.2 Novel Object Recognition (NOR) tasks

There was no statistically significant difference when comparing the number of explorations of the novel object and the familiar object in all experimental groups except 15 minute HI group which was fed with control diet (Figs. 5.8 A and B). No statistically significant difference in delta values (explorations to new object minus familiar object) was observed between the groups (Figs. 5.8 C).

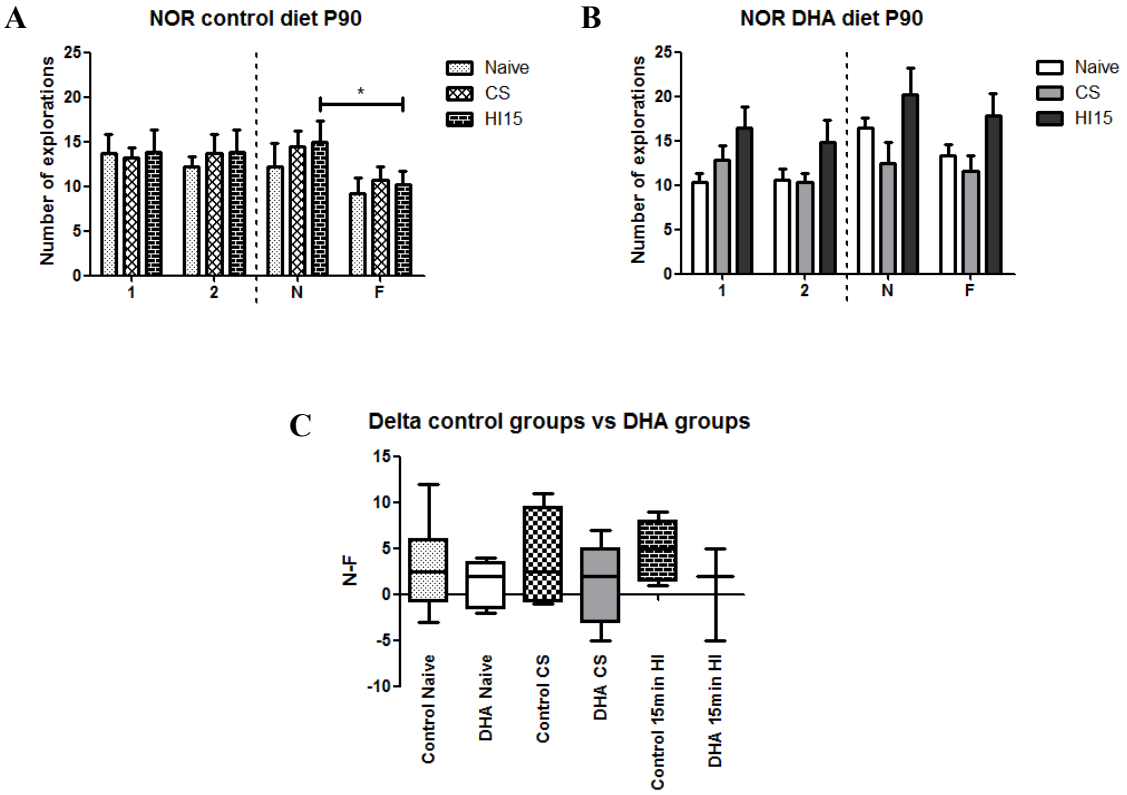


Figure 5.8. NOR test. A and B. The number of explorations of each object in naïve, CS and HI 15 min groups of two different experimental diets, control diet (**A**) and DHA diet (**B**). Data were analysed using Student t-test. If the data were parametric, paired t-test was used; if data were non-parametric, Wilcoxon matched pairs test was used. Data are expressed as mean ± SEM **C.** The delta values (explorations to new object minus familiar object) of different groups of animals which were fed with control and DHA diets. When comparing data between animals in one diet group, data were analysed using one-way ANOVA test with Bonferroni post test if data were parametric and Kruskal-Wallis test and Dunns post test if data were nonparametric. When comparing animals in the same surgical groups between two different diets, data were analysed using Student t-test. Data are expressed as minima and maxima; (control diet: naïve, n=6; CS, n=4; HI 15min, n=5; DHA diet: naïve, n=7; CS, n=9; HI 15min, n=5). *p<0.05.

5.6 Discussion

Many studies have focussed on the neuroprotective properties of n-3 fatty acids in the central nervous system (CNS). In humans, epidemiological studies have shown the benefits of maternal DHA intake with improved offspring outcomes [107, 109, 110, 247]. However, some studies claimed that high DHA intake during latter half pregnancy and lactation showed no difference in children's neurodevelopment, visual acuity or growth between placebo [111-114]. This study examined whether DHA maternal supplementation could have a neuroprotective effect and involved giving a 1.5% DHA enhanced diet to dams from E2 to P21. On E18 the animals were subjected to HIE. According to the information given by the manufacturer, the DHA enhanced diet consisted of 1.5% DHA. However, fatty acid analysis of the DHA enhanced diet in our hands showed that this level was only 0.8% DHA. This could be a consequence of oxidation or other reasons resulting in a loss during or after manufacture, transport and storage of the DHA enhanced diet. We stored both the control and the DHA enhanced diets at -20˚C for up to one year and only defrosted small amounts to feed the mice daily.

It is well accepted that fatty acids levels in plasma reflect the tissue fatty acids levels after a short term dietary intake while the levels in RBC indicate the long term dietary intake. Therefore, in order to identify the maternal fatty acid status analysis of the plasma and RBC's should be undertaken [248-251]. In our study, both the short and long term dietary intake were reflected by the higher DHA levels seen in the plasma and RBC's of the DHA enhanced diet groups. This increase of DHA was also seen in the pup brains of these DHA enhanced diet groups. These results indicate that part of DHA accumulated in the maternal RBC while some of the DHA was transferred to pups through maternal plasma. While it would have been interesting to determine the level of DHA in pup blood, difficulties in sampling meant this was not possible to achieve. However, DHA would most likely accumulate in brains and

enhance cell function [89]. In P1 pup brains of the dams fed with a DHA enhanced diet, the increase of DHA reached 8.26% when compared to control diet. Moreover, when comparing the DHA elevation in the DHA group with the RM1 diet group, the DHA group was 3.89% higher (RM1 diet group: 8.67 ± 0.5%). These data showed that the DHA enhanced maternal diet could alter the DHA content in the offspring brain.

In a study carried out in Japan, a DHA enhanced maternal diet (with 10% DHA as a proportion of total fatty acids) was used with Rice-Vannucci rat model and their data indicated that the DHA enriched maternal diet was neuro-protective [252]. In their study, the DHA level in the DHA group pup brains was only 2.8% greater than the control group (control diet group: 11.2 ± 0.2%; DHA enhanced diet group: 14.0 ± 0.3%) at P7. In both their study and the present study, the DHA level reached around 13% in the offspring brains with regard of the proportion of total fatty acids and yet their study the difference between the DHA enriched diet and the control diet was 5.46%, impling the existence of an alternative source of DHA.

Other than direct ingestion, DHA could also be synthesised from its precursor (ALA) [253, 254]. However, animal and human studies have shown that DHA in the maternal diet is more efficient than ALA in increasing the DHA level in maternal blood, tissue and milk [255, 256]. In our study, the level of ALA (0.08% as a proportion of total fatty acids) was the same in both the control and DHA enhanced diets. Considering the low level of ALA in both diets and the lower efficiency ALA being transferred into DHA, the huge difference of DHA seen in the pup brain in DHA enhanced group was mainly due to the DHA added to the diet. Previous studies have shown that an increase level of DHA in brain is usually accompanied by a decrease in the level of AA [257]. This was reflected in our results.

After proving the DHA level was increased in the brains of the offspring after the maternal DHA supplementation, the HIE model established previously was used to determine if and how the DHA enriched diet improve short- and long-term offspring outcome after HIE. Firstly, the pups' survival rate and their short-term behavioural changes were investigated. The survival rates of the CS and HI groups having the DHA enhanced diet were 2.9% and 6.9% higher respectively when compared to the groups having the control diet. However, these changes were not in statistical significance. But when these data were compared with the previous results when the HIE was establish, it is interesting to see that the survival rates of CS groups of both experimental diets remained at a similar level to previous findings (98.3%); while survival rates of the two HI groups were much lower (about 20% lower) than our previous findings. These results indicate that the diets generally had a poor effect on the HI groups and this effect could barely be improved by the extra DHA in the DHA enriched diet.

Studies that used the rat Rice-Vannucci models combined with DHA maternal supplementation or intra-peritoneal injection of DHA had no reports on the pup survival rate but had other data to support DHA had improved the outcomes after having HIE [252, 258]. The overall appearance of neonates was similar to that described previously in Chapter 3 with one exception: the skin colour. The skin colour of all pups born after the 10% fatty acid maternal diets (the control and the DHA enhanced diets) was more yellow / orange than the those having the general laboratory diet (RM1 diet) which may indicate neonatal jaundice. After 1-2 days the skin colour returned to the normal pink. In a study which used the same maternal diet as was used as control in this study, skin colour change or neonatal jaundice was not reported [156].

Previous PCR data in our experiment indicated that inflammatory cytokines might play an important role in the HI damage in the pup brains. Studies using different cerebral ischaemic

in vivo and *in vitro* models have reported that DHA treatment has an anti-inflammatory effect by reducing inflammatory related factors such as IL-1β, IL-6 and NF-κB [115, 117]. Our data showed there was no significant difference in the IL-1β level in the pup brains regardless which diet was used during the maternal period. This could be due to the low dose of DHA in the DHA enhanced diet in this study although this resulted in a nearly 3-fold increase in DHA in the pup brain.

HIF-1α is a transcription factor that responds to changes such as decreasing oxygen concentration in the cellular environment. Studies using neonatal stroke animal models have demonstrated the HIF-1α protein level peaked at 3 hours [220] and 8 hours [221] from the initial hypoxic insult. However, the durability of HIF-1α protein and gene expression (8-24 hours after ischaemia) has been noted in global ischemia [222, 223]. Because of this, P1 and P3 were chosen to investigate HIF-1α levels. Our data show that at P3 HIF-1α level in DHA HI group was significantly lower than the control group which indicated that DHA diet may improve the hypoxia condition in the pup brains. It has been noted in our P1 data, HIF-1α levels in DHA CS group was significantly higher than DHA naïve and DHA HI15 groups. One possible explanation is those pups who were severely hypoxic would have been died after 15 minutes of hypoxia while those who survived had a higher tolerance level of the hypoxic condition. However, at P3 this trend did not continue and was actually reversed. Other than indicating the hypoxia in the cellular environment, HIF-1α is also known to play important roles in apoptosis [259-261].

Studies using different animal models indicate that apoptosis accounts for much of the neuronal cell death after HIE [168, 262-264]. Studies have shown that DHA and its mediator NDP1 have the effect of reducing apoptosis by altering the levels of apoptotic genes such as Bcl-2 and Bax [265, 266]. Although as mentioned previously in Chapter 3, apoptosis has not been proved to be contributed to the neuronal cell death after in this HIE model, many other

studies claim that apoptosis is a key factor [153, 182, 267, 268]. Caspase-3 data in this study indicate that DHA may reduce the apoptosis, however it may indicate that those pups could not survive after CS regardless it was under hypoxic condition or not had severer neuronal cell death. On the other hand, the higher phospho-bad expression in the DHA diet groups suggests that DHA induced higher levels of pro-apoptotic factor.

As previously mentioned, there are different possible mechanisms behind the neuroprotective effects of DHA. Although our short-term cell data did not provide strong evidence of whether DHA could reduce inflammation and apoptosis, we proceeded to determine the longer term neuroprotective effect of DHA by subjecting the animals to the T-maze alternation and novel object recognition tasks (NOR). T-maze spontaneous alternation task is used to test the working memory, which relies on good functional ability of the hippocampus [269, 270]. In this study, there was no significant difference between naïve, CS and HI group fed with control diet while in the DHA enhanced diet groups the alternation rate of the naïve group was higher than the CS group. These data indicate that the DHA enriched diet may improve the long-term outcome of the naïve group of animals. Ideally, the sample numbers need to be increased to be able to come to a firmer conclusion.

NOR has been widely used to test working memory [160, 176]. In the NOR task, a larger sample number was used. In our previous data, when the model was first established, mice from the naïve group devoted more time to the novel object while the CS group had an equally poor performance when compared to mice from the HI group. However in this study, there was no evidence to show that DHA maternal diet had a neuroprotective effect.

In conclusion, these data suggest that higher maternal dietary DHA is transferred to the pup brains. We show that a maternal diet with an actual DHA content of 0.8% could lead to an increase of DHA level in offsprings. However, the greater mortality rates in both CS and HI

groups regardless of whether they were on control or DHA diets indicated that there might be other issues related to the diet which influence neonatal death rates. The high mortality rate may also reduce the ability to establish HIE in the remaining live pups. Overall, no clear neuroprotective effect was shown for the DHA enriched diet group.

Chapter 6. General Discussion

6.1 Discussion

Hypoxic ischaemic encephalopathy (HIE), also known as perinatal asphyxia, is one of the most common causes of neonatal death and long-term neurological sequelae. However, other than hypothermia there is no effective treatment for the damaged brain. Recently, many studies have focussed on the neuroprotective properties of n-3 fatty acids in the central nervous system (CNS). In humans, epidemiological studies have shown the benefits of maternal DHA intake with improved offspring outcomes [107, 109, 110, 247]. However, some studies claimed that high DHA intake during the latter half of pregnancy and lactation showed no difference in the neurodevelopment of children, visual acuity or growth when compared to placebos [111-114]. In order to test potential treatments for HIE, many animal models were establish to mimic the HIE in human. The most common animal model for HIE is the Rice-Vannucci model, which involves a unilateral common carotid artery ligation on a 7-day-postnatal rat pup followed by 4 to 8 hours of hypoxia. This model has successfully created infarctions in the brain and has been used to investigate the mechanisms behind HIE. However, creating the hypoxic and ischaemic conditions locally in a newborn animal without considering its mother's situation is not clinically relevant. With the aim of testing whether a maternal DHA intake would have a neuroprotective effect against the HIE in the offspring, a true perinatal asphyxia animal model is needed. Therefore, an adaptation of the intrauterine HIE rat model developed by Bjelke *et al* [152] was attempted and this study is the first one to establish a true intrauterine model on mice.

In the present study, we found that pups exposed to 15 minutes of HI had a worse short term outcomes than pups in the naïve, CS and 10 minutes HI groups when the 5 criteria of Apgar scores were considered. Thus, 15 minutes of HI was chosen for further verifying the HIE model. As several human studies have shown that cytokines play an important role in the brain damage associated with HIE [190-192], in the current study, the level of different pro-

inflammatory and anti-inflammatory genes expression were measured. The difference in these genes expression between the naïve and CS groups were compared to see whether there was any effect of C-section itself and natural birth. The comparison between CS and HI groups was also performed to determine the damage after introducing HI to the pups. The mRNA levels of inflammatory markers (especially the IL-1β) indicated that inflammation was present from an early stage in brains subjected to HI. However, there was a tendency for these genes to increase in the CS group when compared with the naïve group. This may be a consequence of the delivery method. When mice are delivered vaginally the mother is able to revive pups individually as each pup is delivered. However, it may be that in the CS delivery model, pup resuscitation is compromised as all pups are delivered at once. Studies using intrauterine rat HIE model has shown NF-κB p65 mRNA levels expression increased after HI damage [271]. However, in this study, although activation of NF-κB at different covalent modification sites was examined (phosphorylation at Ser468 and 536 and acetylation at Lys310), none of them have showed a positive result. Apoptotic cell death plays a key role in neurodegeneration after HI brain injury [163]. Caspase-3 activation, Bcl-2 family of proteins expression and TUNEL assay are validated markers of neuronal apoptosis and has been reported to show an up-regulated expression in the rat intrauterine HIE model [267, 268, 272]. In this study we did not find any significant difference between the groups with respect to the caspase-3 activity, Bcl-2 family expression and apoptotic bodies in TUNEL assay in pup brains. However, the caspase-3 expression tended to correspond with the length of the hypoxia generated. The histological evidence was then investigated using cresyl violet and H&E stainings whereby unhealthy nuclei and reduction in layers of neurons in hippocampus area were observed in the mice that had HI. In some of the CS group mice brain, the unhealthy neurons appeared as well. However, these findings were not observed consistently when the n number was 6 in each group. This might become statistically significant if the

experiment was carried out with a larger number of animals. Since the long-term cognitive dysfunction is the most significant consequence of HIE, water maze, T-maze alternative tasks, fear conditioning and NOR were performed to further confirm the validity of this intrauterine HIE mouse model. These data indicated that all the animals involved had no motor deficiency, animals in the CS and 15 min HI groups had worse long-term outcomes than those in the naïve group but their performance were equally bad.

When the model was still being verified, the DHA maternal diet supplementation was tested due to the limited time of the project. As seen in the results, the DHA was successfully transferred into the pup brain via maternal diet intake. However, the DHA did not improve the survival rate of the HI pups. At the cellular level, DHA enhanced diet + HI group showed a decrease in HIF-1α and phospho-p38 levels when compared with control diet + HI group. This may suggest that there is a neuroprotective effect of DHA against HIE. The long-term behavioural results did not indicate a better or a worse outcome in the offspring by adding extra DHA in the maternal diet. This may have been due to the low percentage of DHA content, but the manufacturer had confirmed that by adding any more DHA into the diet would have made the diet too oily and difficult to produce. Overall, there was not a clear conclusion of whether adding 0.8% of DHA in maternal diet would provide neuroprotection against HIE. It should also be noted that in fact, both control and the DHA enhanced diets showed a significantly lower survival rate in pups that suffered 15 minutes HI than the previously used experimental diet supplied by the animal facility.

Another part of work in this study was to investigate whether systemic maternal inflammation would lead to a worse outcome for newborn that were exposed to HI. The serotype, dose, timing and route of LPS injection may lead to different types of inflammation resulting in different outcomes. In this study, systemic inflammation was induced successfully using LPS. An increase in the stillbirth rate was observed suggesting a worse

outcome for the fetus. Many inflammation-related chemokines and cytokines were highly expressed in maternal tissue samples. However, when this inflamed condition was combined with HI, the survival rate of the HIE model did not show any difference compared with the no injection vehicle. The only celluar change seen in the data was the up-regulation of p38 in the pup brains in the LPS + HI group. P38 is a key factor in both inflammation and apoptosis, thus the increase in p38 may indicate that LPS had an adverse effect to the neonates. However, other cellular and long-term outcomes did not indicate a worse result, although in some of them it did lead to an opposite trend. Although a failing in this part of the study was that the control group was not ideal.

In conclusion, the inconsistent and conflicting data may indicate that 15 minutes is probably too long for observing significant and extensive brain damage in the surviving pups. Hence the 15 minutes intrauterine HIE mouse model did not fully mimic what happens in human. As for the DHA enhanced diet, the study showed some evidence of neuroprotection but at this stage this is not a solid conclusion to be made. The LPS induced inflammtion in late pregnancy in mice led to increased fetal mortality and apoptosis in postnatal pup brains, but did not worsen the long-term outcomes of those pups when further exposed to HI.

6.2 Future work

For verifying the HIE model:

- Increase the n number to at least 12 based on the experience of Hagberg's group who use the Rice Vannucci model.
- Explore more of the secondary injury of HIE, such as the accumulation of Ca^{2+} and NO in mitochondria.
- Explore more of the tertiary phase of brain injury.

For the extension of the HIE model - maternal systemic inflammation during late pregnancy study:

- Increase the n number of saline injection group and use it as the control group.
- Test different causes of maternal inflammation/infections: bacteremia, urinary tract infections and chorioamnionitis.

For potential treatment of HIE:

- Test different concentrations of n-3 PUFAs in the maternal diet.
- Use other delivery methods of n-3 PUFAs for example oral gavage or i.p. injection.
- Test other potential treatments or combined other potential treatments with a DHA enriched maternal diet (such as but not limited to hypothermia, anaesthetics and stem cells).

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