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ON EVALUATION OF ORGAN DAMAGE IN PERINATAL ASPHYXIA: AN EXPERIMENTAL AND CLINICAL STUDY

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To Kamilla, Malva and Lisa

*“Writing is easy. You only need to stare at a piece of blank paper until
your forehead bleeds”*

Douglas Adams 1952-2001

ABSTRACT

Leakage of intracellular enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) signalling multi organ dysfunction (MOD) is seen together with hypoxic ischemic encephalopathy (HIE) after perinatal asphyxia. Hypothermia (HT) can attenuate HIE following perinatal asphyxia and hypoxia-ischemia (HI) but the neuroprotective efficacy falls dramatically the longer the delay in initiating HT.

However, the knowledge was scarce regarding protective or adverse effects of HT in organs beyond the brain. Also, the relative effectiveness of the two clinically used modes of cooling; selective head cooling (SHC) and whole body cooling (WBC) had not been studied.

In this thesis we studied (1) the time course of LDH, ALT and AST serum activity during the first week of life after asphyxia and (2) if these enzymes predicts HIE in newborn term infants with asphyxia and intra partum signs of fetal distress. (3) In a newborn pig model of global HI, we investigated whether plasma values of these enzymes differ between newborn pigs with liver injures after a severe global HI insult and pigs with normal livers. (4) In the newborn pig model of global HI we studied whether 24h HT initiated 3h after global HI is brain- and/or organ-protective using pathology, neurology and biochemical markers.

RESULTS

(1) Two different temporal serum-ALT or AST pattern were seen in 26 newborn asphyxiated infants during the first week post partum. One pattern were characterized by increasing values during first days of life while the other pattern showed a peak enzyme activity seen in the first sample at less than 12 hours after birth followed by a decrease. (2) In asphyxiated infants with differing degree of HIE (n=44) and in infants where there had been signs of fetal distress during birth (n=202) a cut off level of 1049 U/L for LDH was the most suitable predictor of mild, moderate and severe HIE with a sensitivity of 100% and specificity of 97%. The cut off

levels for ALT and AST were 16 and 59 U/L respectively giving a sensitivity of 100% and 93% and a specificity of 83% and 93% respectively.

(3) No differences in area under the curve (AUC) for AST, ALT and LDH values were seen between newborn pigs with liver injuries (n=12) after a severe global HI insult and pigs with normal livers (n=7) during 72h after a HI insult. However, in pigs with liver injuries a transient significant increase in LDH at the end of the HI insult (928 U/L (567-1031)) was seen compared to the baseline value (679 U/L (548-866), p=0.010). (4) No differences in injury to the brain or organs in pigs subjected to global HI followed by NT (n=16) or 24h of SHC (n=17) or WBC (n=17), initiated 3h after the insult were seen. The post-insult decline in lactate was temperature independent. However HT animals normalized their plasma-calcium, magnesium and potassium significantly faster than NT. There were no differences in adverse effects across groups.

CONCLUSIONS

There is a potential usefulness of intracellular enzymes as predictors of HIE in newborn infants with perinatal asphyxia. In the newborn pig subjected to global HI only LDH increases in pigs with pathological changes in the liver and normal values of ALT and AST do not exclude hepatic injury. Delayed SHC or WBC, initiated 3h after HI; do not reduce pathology in the brain nor in organs in newborn pigs after global HI.

Key words: perinatal asphyxia, hypoxia-ischemia, hypothermia, pig, lactate dehydrogenase, aminotransferases,

ABBREVIATIONS

aEEG, Amplitude integrated EEG

AF, Amniotic fluid

ATP, Adenosine Triphosphate

AUC, Area under curve

CFM, Cerebral function monitoring

CO, Cardiac output

CTG, Cardiotocograph

ECG, Electrocardiogram

EEG, Electroencephalography

EFM, Electronic fetal monitoring

GGT, Gamma glutamyltransferase

HI, Hypoxia-ischemia

HIE, Hypoxic Ischemic Encephalopathy

HT, Hypothermia

I/R, Ischemia/reperfusion

IL-6, Interleukine-6

MOD, Multi Organ Dysfunction

MRI, Magnetic Resonance Imaging

NO, Nitric Oxide

PCr, Phosphate Creatinine

Pi, Inorganic phosphate

ROC, Receiver operating characteristic

SHC, Selective Head Cooling

TMI, Transient Myocardial Ischemia

WBC, Whole Body Cooling

TNF α , Tumor necrosis factor-alfa

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on studies reported in the following papers, referred to in the text by their Roman numerals

- I. Mathias Karlsson, Mats Blennow, Antal Nemeth and Birger Winbladh. Dynamics of Hepatic Enzyme Activity following Birth Asphyxia. *Acta Paediatrica*. 2006; 95:1405-11.
- II. Mathias Karlsson, James R Tooley*, Saulius Satas, Catherine E Hobbs, Ela Chakkarapani, Janet Stone, Helen Porter, Marianne Thoresen. Delayed hypothermia as selective head cooling or whole body cooling does not protect brain or body in newborn pig subjected to hypoxia-ischemia. *(The authors Karlsson and Tooley have had equal contribution to the paper). *Pediatric Research*. 2008 in press
- III. Mathias Karlsson, Saulius Satas, Janet Stone, Helen Porter, Marianne Thoresen. Liver enzymes cannot be used to predict liver damage after global hypoxia-ischemia in a neonatal pig model. (Submitted)
- IV. Mathias Karlsson, Eva Wiberg-Itzel, Ela Chakkarapani, Mats Blennow, Birger Winbladh, Marianne Thoresen. Lactate dehydrogenase predicts hypoxic ischemic encephalopathy in newborn infants. (Submitted)

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1 INTRODUCTION AND BACKGROUND

About four million newborn children die annually. Almost one quarter (23%) of these deaths are caused by perinatal asphyxia (3) (Figure 1), most of them in developing countries. In addition, perinatal asphyxia causes an even greater number of children to develop neurological sequelae. The clinical signs following perinatal asphyxia have been called hypoxic ischemic encephalopathy (HIE). The onset of the asphyctic event leading to HIE could start antenatally or close to/during birth and the reported incidence of asphyxia with antenatal vs. perinatal onset differs substantially between studies (4-6). The diagnostic and predictive methods for severe asphyxia and HIE during labour and postnatally are focused on the condition at birth typically assessed by Apgar Scores and acute laboratory changes (blood gases) and supplementary methods for diagnosis and prediction of antenatal and non-acidotic prolonged asphyxia are lacking.

The defence mechanisms when a fetus is exposed to hypoxia-ischemia (HI) is similar between species (7, 8). This mechanism is based on the ability to centralize cardiac output to prioritised organs such as the brain, heart and adrenals at the expense of, for the moment, less important organs such as the liver, lungs, skin and muscles. Multi-organ dysfunction (MOD) is a natural consequence of this defence mechanism (8) because of the cellular damage inflicted on the non-prioritised organs. Injured cells leak intracellular enzymes, some of which are able to be measured in plasma e.g. lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Previously it has been reported that:

- Increased levels of LDH, AST and ALT are seen after neonatal asphyxia (9-11) as early as in the cord blood directly after birth. (10, 12).
- LDH, ALT and AST rise substantially after organ damage (9, 12-14).

- The elevations in LDH, ALT and AST are seen as long as 7-10 days after the onset of HI (10, 13, 14)

Hence, these enzymes that leak into the circulation after cellular damage in organs affected by HI may be used as potential predictors of timing and grade of the hypoxic-ischemic injury in both the perinatal period and in infants with ante partum asphyxia.

Hypothermia (HT) after hypoxia-ischemia (HI) is neuroprotective in experimental models across different species (15-17). Recently multicenter, randomized controlled clinical trials used two cooling methods; selective head cooling (SHC) combined with mild systemic hypothermia (18) or whole body cooling (WBC) (19, 20). Both methods offered protection and this is the first time any intervention has improved outcome in infants suffering HIE. It has been suggested that SHC is superior to WBC, offering greater cortical protection and fewer adverse systemic effects; however there is no trial data to support this suggestion. The two modes of cooling have not been compared “head to head” in the same clinical trial and this question is eminently suitable for an experimental study like we have undertaken,

1.1 Asphyxia & HIE

Every hour, 104 children die as a result of asphyxia (3). That is approximately 8% of the total global paediatric mortality (age less than five years) making it a serious problem. Due to a lack of resources developing countries are worse off. Yet this is a global issue requiring urgent attention.

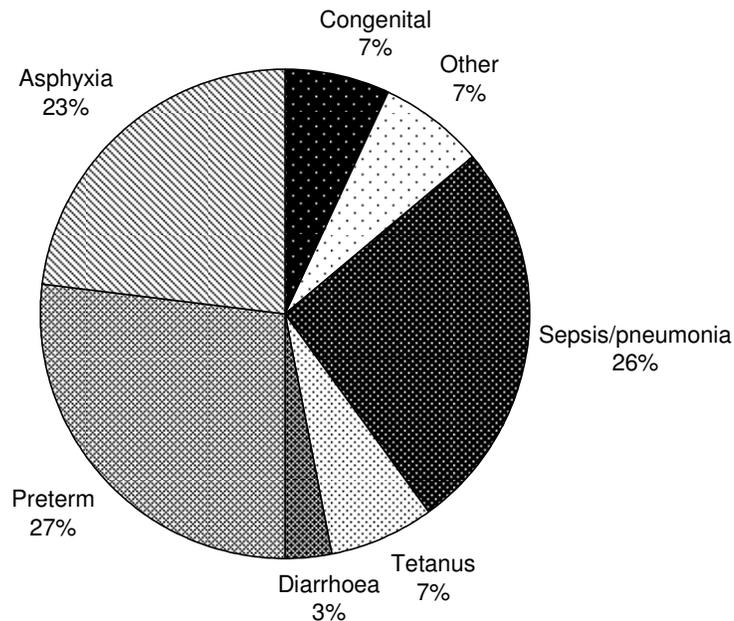


Figure 1 Global causes of death in the neonatal period, (Adapted from Lawn 2005) (3).

1.2 Definitions

1.2.1 Hypoxia-Ischemia

The term Hypoxia-Ischemia (HI) is frequently used. Hypoxia means a partial or complete oxygen deficiency while ischemia means decrease in blood flow. Reasons for ischemia could be insufficient cardiac output, hypotension or vascular occlusion. While ischemia is one important cause of hypoxia it could also occur without a decrease in blood flow due to inadequate oxygenation or severe anemia. HI therefore refers to a condition involving both components. Anoxia is defined as a complete lack of oxygen delivery to the tissue.

1.2.2 Asphyxia

In the newborn, asphyxia means a disruption in pulmonary or placental gas exchange leading to acidosis. The acidosis is both respiratory (due to the decrease in gas exchange) with a raised pCO₂ level and metabolic (accumulation of lactic acid). There is more than one criterion for diagnosis of asphyxia. One set of criteria for defining asphyxia that could be linked to adverse outcome (e.g. cerebral palsy) is the consensus from the American Academy of Pediatrics and the American College of Obstetrics and Gynaecology from 1992 shown in Box 1. These criteria are similar to other statements (21) and are to be seen as tools when defining groups for research purposes

| |
|---|
| Arterial pH of less than 7.00 |
| Apgar score of less than four for longer than 5 minutes |
| Neonatal neurologic sequelae |
| Multi-organ system dysfunction |

Box 1 Criteria for intrapartum asphyxia from the American college of obstetrics and gynaecology (1)

1.2.3 HIE and NE

Neonatal encephalopathy (NE) is a clinical syndrome with neurological dysfunction the first days of life in term neonates, often including seizures (22). The etiology is multiple and birth asphyxia is the cause in approximately 28% of the infants developing cerebral palsy after NE (23). Several terminologies are used for the same condition and the most commonly used term is “hypoxic ischemic encephalopathy” (HIE) suggesting hypoxia and/or ischemia as the etiology (22). HIE is often graded 1, 2 or 3 based on symptomatology. This severity grading shows reasonable correlation to prognosis. The grades of HIE and the relationship to poor outcome is shown in Table 1. Moderate to severe HIE was seen in 3.8/1000 term infants in an

Australian study (5) and was followed by cerebral palsy (CP) and death in most cases (24). The definition and incidence of asphyxia and HIE differs between countries and studies. In Sweden and United Kingdom, asphyxia, defined as an Apgar score less than 7 at five minutes post partum, asphyxia will occur in about 7/1000 term births leading to 1.8/1000 infants being born with HIE (25, 26).

| Grade | HIE 1 (mild) | HIE 2 (moderate) | HIE 3 (severe) |
|-------------------------------|--|--|--|
| Symptoms at 24h of age | Irritability Mild hypotonia Poor sucking | Lethargy Abnormalities in tone Requires tube feeding | Comatose Severe hypotonia Failure of spontaneous respiration |
| Seizures | No | Yes | Yes |
| Poor outcome (%) | 1.6% | 24% | 78% |

Table 1 Grades of hypoxic ischemic encephalopathy (HIE) and percentage with bad outcome defined as death or severe handicap of infants with HIE (27, 28)

1.3 *Clinical symptoms of perinatal asphyxia*

1.3.1 Symptoms of asphyxia in the fetus

The lungs of the fetus in utero do not perform gas exchange and the fetus is therefore dependent on the oxygenation of its mother, the placental perfusion and the difference in the oxygen binding capacity between the fetal and the maternal hemoglobin. Normally these mechanisms give the fetus enough oxygen for its requirements. The fetal circulation differs from that of adults and has a low pulmonary circulation (29), and a prioritized circulation to the brain from the umbilical vein by shunting blood away from the portal circulation through the ductus venosus and away from the pulmonary circulation via foramen ovale to the left atrium and ventricle.

1.3.1.1 The fetus during normal labour

When the uterus contracts during labour the blood supply to the maternal placenta is almost interrupted (30) causing a transient decrease of oxygen in the fetal blood. Normally, a healthy fetus uses multiple mechanisms to compensate for this fall in oxygen levels as listed below:

- The high concentration of hemoglobin, mainly of fetal type, makes the fetal blood a good carrier for oxygen
- High cardiac output (CO)
- Redistribution of cardiac output

The redistribution of cardiac output to prioritised organs like brain, heart and adrenals at the expense of less important organs like the liver, lungs, skin and muscles (31) (Figure 2) is in common to different species (7, 8). Also within the central nervous system (CNS) a redistribution of the blood flow to the brain stem and subcortical areas is seen during hypoxia in fetal lambs (32).

In the absolute majority of all births, the fetal strategies to cope with transient hypoxia during labour are sufficient. In a situation where the oxygen supply is limited the fetus produces energy by anaerobic metabolism. This means that pyruvate, instead of entering the citric acid cycle, is reduced to lactate and H^+ . An anaerobic metabolism needs almost 20 times more glucose to maintain the same energy production as the aerobic metabolism. This means that in a situation where the fetus enters the labour with low or empty glycogen reserves, due to chronic placental deficiency, preterm labour or exhaust of these reserves during prolonged labour could cause illness of the fetus. Also, in a healthy fetus, with an antenatal period without complications, a prolonged or substantial decrease in oxygen supply could be enough to cause damage to the fetus.

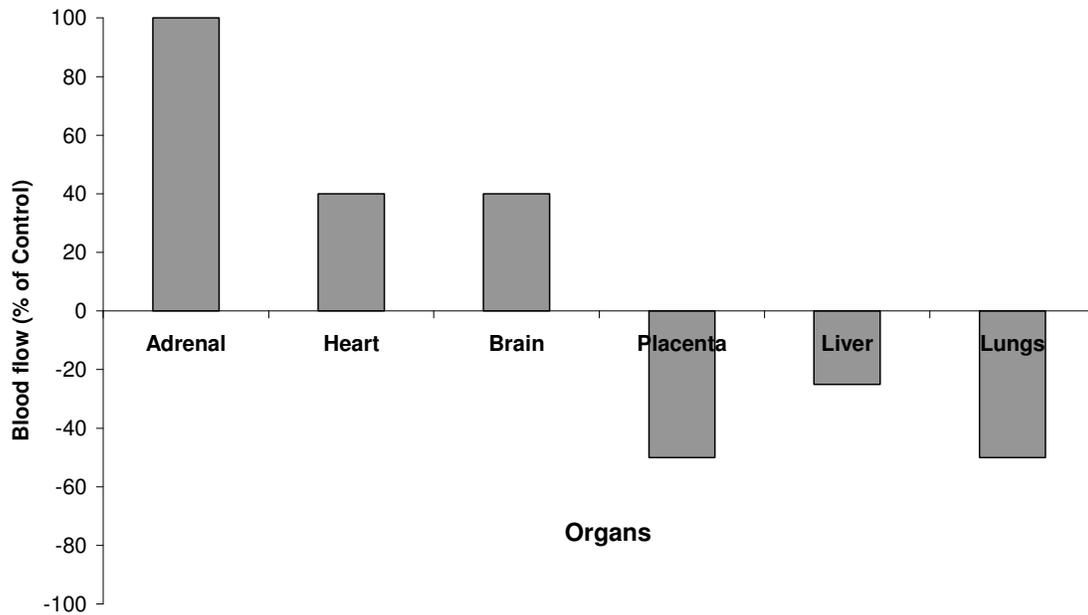


Figure 2 Redistribution of cardiac output during HI in fetal lamb (33) Methods for detection of ante-and intrapartum asphyxia detection. Adapted from Itskovitz J 1987

Clinical risk scoring.

If the fetus has been subjected to chronic hypoxia and/or insufficient nutrition for a longer period of time this could be reflected by growth retardation and decrease in amniotic fluid which can be detected by ultrasound (34). During birth, meconium stained amniotic fluid (AF) might be a acute sign of fetal distress even if only small differences in cord pH and Apgar score are seen between infants with meconium stained AF and infants with clear AF since passing of meconium is quite common in full and post term non asphyctic fetuses (35). Significant intrapartum predictors of neonatal encephalopathy in an Australian case-control study were maternal pyrexia, persistent fetal occipitoposterior position and acute events during birth (5). However, the sensitivity for using clinical risk scoring as a predictor of fetal asphyxia is low with a positive predictive value of 3% (36) and consequently not sufficient.

Methods for intrapartum surveillance of the fetus

In Sweden four methods are in use for intrapartum surveillance for detection of severe asphyxia and are now briefly described. The sensitivity and specificity of the methods for prediction of mild HIE are listed in Table 2. *Electronic fetal monitoring (EFM)* register the fetal heart rate together with contractions of the uterus using a cardiotocograph (CTG). An abnormal CTG trace could be an alteration in fetal heart rate due to asphyxia. However, when using CTG there is a risk of an increased numbers of operative deliveries due to the high rate of false positives (37). In the method combining CTG with an automated analysis of the fetal electrocardiogram ECG (*ST-analysis (STAN®)*) (38) myocardial ischemia could be mirrored in specific parts of the ECG complex like the ST segment. *Scalp blood: pH or lactic acid concentrations* are frequently used as a compliment to EFM. As previously described, HI can cause acidosis and anaerobic metabolism. Fetal blood is collected with a capillary from the fetal scalp via the vagina and analyzed at point-of-care for validation of the fetal pH or the amount of lactic acid accumulated.

The physiological background to the limited benefits of the use of pH and lactate both in fetal scalp blood and in newborn infants are presented in section 1.8.2.1

| Metod | EFM | pH FSB | Lactate FSB |
|--------------|------------|---------------|--------------------|
| Sensitivity | 54% | 31% | 50% |
| Specificity | 80% | 73% | 76% |

Table 2 The sensitivity and specificity for prediction of mild hypoxic ischemic encephalopathy reported for the methods used for intrapartum surveillance in Sweden (39, 40). EFM, decreased variability on electronic fetal monitoring; FSB=fetal scalp blood

1.3.2 Symptoms of asphyxia in the newborn infant

Many studies during the last five decades have reported Apgar score as a single or combined clinical marker (25, 41, 42) for identification of infants at risk of adverse outcome after perinatal asphyxia.

The 10-point Apgar score presented in 1953 by the anaesthesiologist Virginia Apgar (41) is based on the evaluation of the five assessments: heart rate; respiratory effort; muscle tone; reflex irritability and colour in the newborn. Fifty years later the Apgar scoring system is still a good predictor of survival (42) with a relative risk of 1460 for neonatal death in term infants with a 5min Apgar score of 0-3. However, Apgar score can also be decreased in infants with other conditions, for example anomalies, presence of drugs and preterm birth and the predictive value of Apgar score alone for detection of HIE is insufficient during first hours in life (43)

1.3.3 Clinical symptoms of organ dysfunction in the asphyxiated neonate

As seen in the criteria for diagnosis of intrapartum asphyxia from for example the American Academy of Pediatrics and the American College of Obstetrics and Gynaecology (section 1.2.2) one criterion is multi organ dysfunction (MOD).

The combined criteria for MOD are based on biochemical and clinical measurements and differ somewhat between different studies (2, 44-47). Criteria used by Shah et.al (2) are listed in box 2.

Renal: anuria or oliguria (< 1ml/kg/h) for 24h or more, and a serum creatinine concentration > 100mmol/L; or anuria/oliguria for > 36h; or any serum creatinine > 125mmol/L; or serial serum creatinine values that increase postnatally

Cardiovascular: hypotension demanding treatment with an inotrope drug for more than 24h to maintain blood pressure within the normal range, or electrocardiographic evidence of transient myocardial ischemia

Pulmonary: need for ventilator support with oxygen requirement > 40% for at least the first four hours after birth

Hepatic: aspartate aminotransferase > 100U/L or alanine aminotransferase > 100U/L at any time during the first week after birth

Box 2 Criteria for organ dysfunction in newborn infants with perinatal asphyxia used by Shah et.al 2004 (2).

MOD is a natural consequence of the defence mechanism where the cardiac output is prioritized to the brain, heart and adrenals on behalf of the other organ systems (8, 31, 48). The organ systems involved in MOD are the renal, hepatic, intestinal, pulmonary and cardiovascular systems. Studies suggest that MOD is present to some degree in all infants with HIE (2) with the lungs and the liver as the most frequently affected organs (86 and 85% respectively) followed by the renal (70%) and the cardiovascular systems (62%) (2). These proportions are similar across different studies (2, 44). In a study using mild acidosis (scalp or cord blood pH <7.2) and low Apgar score (<7 at 5 minutes) as the inclusion criteria, at least one organ was affected by the fetal distress in the majority of the infants (82%) (45). As described, hepatic involvement is frequently found in the studies where aminotransferases have been analyzed as the biochemical markers of liver damage. Still, many studies do not include hepatic dysfunction in the multisystem involvement at all or summarize hepatic findings as “gastrointestinal problems”. The reason why the clinical and scientific interest for hepatic dysfunction after HI in the newborn has been scarce could be that the liver dysfunction does not require any immediate treatment and is transient, as far as we know, returning to baseline within 10-14 days. The liver contains 80% of all the macrophages in the human body, and is highly involved in so many metabolic processes that are of importance in research and medical care of newborn infants. As such, to the author, this rather passive relation to hepatic damage is difficult to explain.

1.3.4 Clinical neurological symptoms in the asphyxiated neonate

As previously described under section 1.2.3 the clinical signs following perinatal asphyxia have been called HIE. The severity of HIE is traditionally given a grading of I (mild), II (moderate) or III (severe) that correlates well with neurological outcome (2). The grading is

based on the clinical score first published by Sarnat (22) and later improved by Levene (27) and others.

1.3.4.1 Mild HIE

In the mild form of HIE, no seizures are seen and the EEG pattern is normal (Figure 3a). The infant is conscious but irritable and jittery. The jitteriness is seen together with increased heart rate and dilated pupils as signs of increased sympathetic activity. Reflexes are normal or increased but the sucking can be poor.

1.3.4.2 Moderate HIE

Seizures are commonly seen within 12h after birth and the EEG is abnormal. The infant responds slowly to stimuli and spontaneous movement is scarce. Low heart rate, increased secretions from mucus membranes and constricted pupils are seen and the infant is hypotonic and lethargic.

1.3.4.3 Severe HIE

As for moderate HIE, seizures are frequently seen but are more often prolonged and difficult to treat with anticonvulsive drugs. Seizures occur in 70% of cases having moderate to severe HIE and multiple types are seen including non-convulsive types that can only be detected with EEG. The EEG is abnormal (Figure 3b) with decreased background activity, voltage suppression or in its severest form isoelectric with short burst of high activity. The infant is stuporous with neither reflexes nor spontaneous movement. It is also unable to breathe spontaneously.

←-Hypoxia- 45 min→←Reoxygenation with air-----

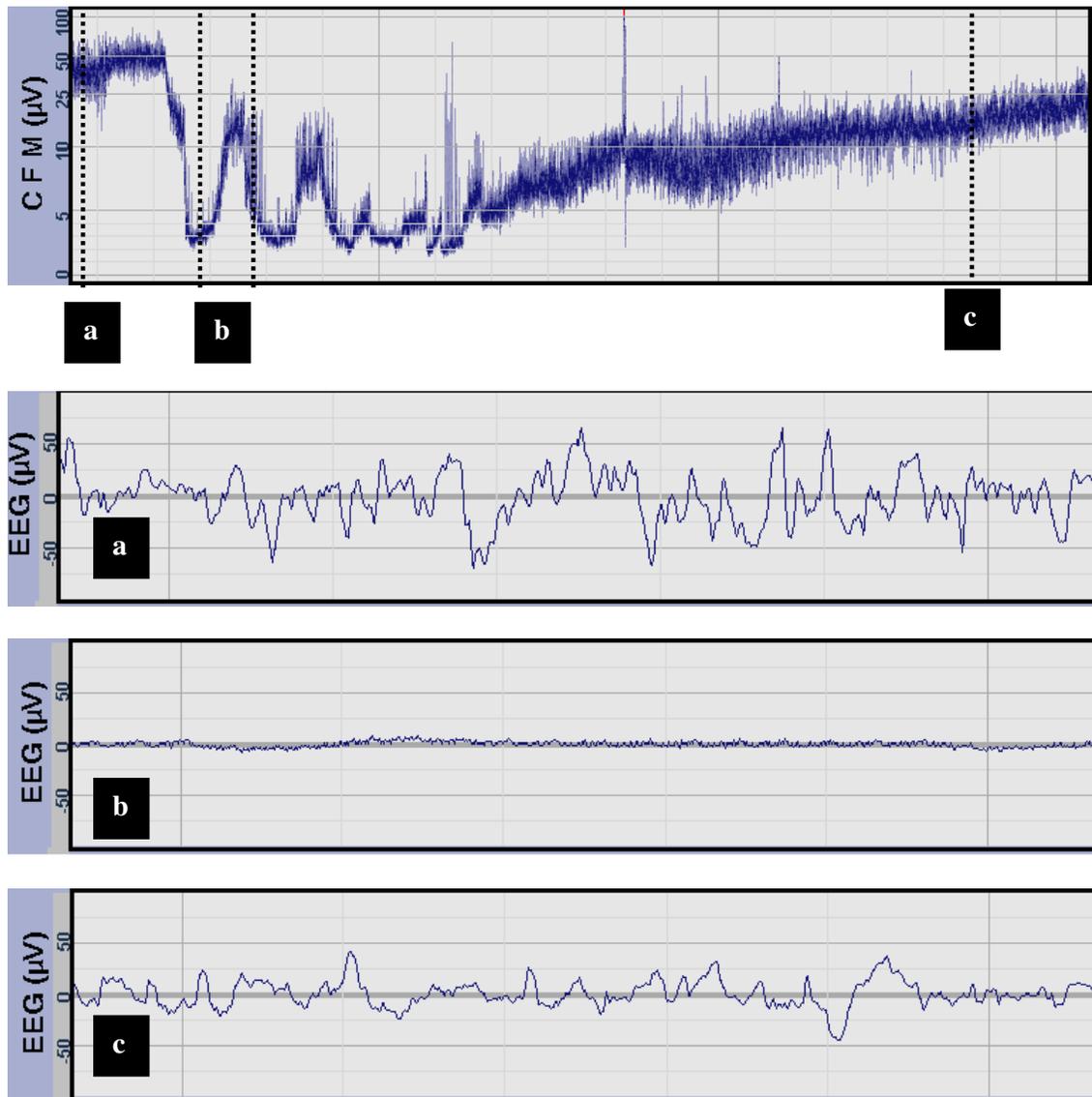


Figure 3 First picture shows a CFM pattern from a 16h old pig under halothane anaesthesia undergoing a 45min long hypoxic insult (ventilation with 5.5% O_2). Arterial Ph was 6.8 and lactate 23mmol/L after the 45 min insult. The pig was reoxygenation was with air and SaO_2 and MABP was kept normal thereafter. Picture A-C shows the EEG from the three marked sequences on the CFM. A: baseline EEG under halothane anaesthesia, B: five minutes after start of hypoxia, the EEG is flat. C: 100 min after reoxygenation showing some recovery of the EEG

1.4 *Pathophysiology of asphyxia: General aspects*

1.4.1 Cell damage

When a cell is exposed to HI the outcome depends of the degree and duration of the insult. If the insult is brief the cellular injury may be reversible. However, if the HI is severe enough the cell will be irreversibly damaged and die. The two main, known routes leading to cell death are necrosis and apoptosis. The etiological and molecular mechanisms leading to one or the other are different in many aspects while others are present in both. Briefly necrosis is seen after loss of blood supply to the cell but also if the cell is exposed to different kinds of toxins. Apoptosis is seen both under normal and pathological conditions (49). Examples of normal conditions are the regulated cell deaths seen during embryogenesis and the development of the neonatal brain (50). The general main actions in the pathophysiological mechanisms of cell death are presented in figure 4 and described in more detail below.

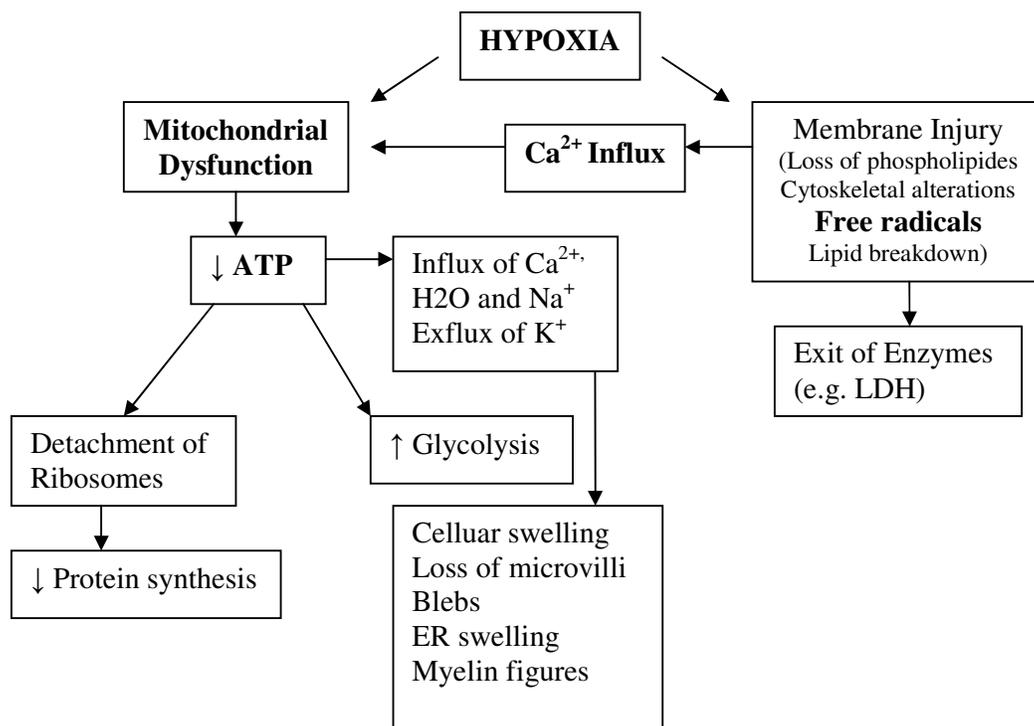


Figure 4 Schematic summary of mechanisms involved in hypoxia-ischemia induced cellular injury. Adapted from Robbins Basic Pathology (Kumar et al. 2007) (49). ER, endoplasmic reticulum, LDH, lactate dehydrogenase.

1.4.1.1 ATP depletion

The mitochondria are the intracellular organelles responsible for energy production by oxidative phosphorylation. This energy is carried by adenosine triphosphate (ATP). HI damages the mitochondria through calcium accumulation, oxidative stress, and breakdown of membrane phospholipids as explained in the following sections. This in turn results in disturbed membrane potential and inhibited oxidative phosphorylation (51). The mitochondria contain cytochrome C (as a part of the electron transport chain) that leaks out if the mitochondria membrane is damaged. Cytochrome C is also known to participate in the pathway leading to apoptosis (49). The lack of oxygen during HI makes it impossible to

produce an adequate amount of ATP (52) needed for normal cellular functions. When ATP is catabolised to ADP (diphosphate) and AMP (monophosphate), these precursors will accumulate and stimulate the enzyme phosphofructokinase. This enzyme will then increase the rate of anaerobic glycolysis causing depletion of glycogen and accumulation of lactate. Lactate is produced by the reduction of pyruvate by the lactate dehydrogenase enzymatic reaction (anaerobic metabolism) (53) and this results in an intracellular acidosis. The ATP depletion is an early step in the HI induced cell death cascade and impacts upon the following steps.

1.4.1.2 Disturbed electrolyte homeostasis

ATP is needed for the normal transport of sodium (Na) and potassium (K) ions over the plasma membrane by Na^+/K^+ -ATPase (54). Lack of ATP will have negative impact of this transport leading to diffusion of potassium out from the cell and an influx of sodium (55) together with water through osmosis causing cellular swelling.

Influx of calcium into the cell has been known as an important part of the cell death etiology for more than 30 years (56). When considering the 10 000-fold difference in the extra and intra cellular calcium concentration (in favour of the extracellular fluid) it is easy to see that a calcium influx will easily occur if the calcium homeostasis mechanisms, like the ATP-driven Ca^{2+} exchangers are disturbed (for review: Blaustein MP 1988 (57)).

1.4.1.3 Decrease in protein synthesis

Both during and after HI, the protein synthesis in the cell is inhibited by the lack of energy. If insufficient ATP levels are present, as during HI, all steps in building a protein, such as chain initiation, elongation and termination, are blocked. In the post ischemic phase when the energy is present the elongation and termination steps of the protein synthesis are restored.

However, the initiation of new polypeptide formation remains inactive resulting in a prolonged blockage of protein synthesis (58).

1.4.1.4 Free radicals

Besides the fact that HI blocks protein synthesis, it also leads to breakdown of existing proteins. This destruction of cellular proteins is induced by free radicals (59)

Free radicals are atoms or molecules with unpaired electrons on an otherwise open shell configuration. These unpaired electrons are highly reactive and are thus likely to take part in chemical reactions. The increased concentration of cytosolic calcium will change the enzyme ratio in organs such as the liver and lungs in favour of oxidases (60). If oxygen is present, these oxidases will support the production of different free radicals. Free radicals could also be produced in neutrophils, mitochondria and through metabolism of catecholamines and arachidonic acid (61). The free radicals initiate lipid peroxidation of the cellular membrane. This leads to loss of membrane integrity and eventually cell death (62)

The intracellular calcium overload seen during HI will also activate nitric oxide (NO)-synthase. Through its action (NO), citrulline and water will be produced from the precursor arginine (63).

NO may then react with other free radicals forming even more reactive molecules with greater potential to cause cell damage (64).

1.4.1.5 Membrane injury

The mechanism of how the different cellular membranes are damaged during HI is multifactorial and not exclusively induced by the free radicals described above. Calcium influx activates phospholipases that will have negative impact on the membrane phospholipids and proteases that will damage the cytoskeleton.

As the membranes lose their integrity there is a great risk that the damage will become irreversible with massive calcium influx and profound leakage of intracellular enzymes into the peripheral circulation.

1.5 *Intracellular enzymes*

1.5.1 LDH

The lactate dehydrogenase (LDH) enzyme is widely distributed in tissue, particularly in the heart, liver, muscles and kidneys. LDH catalyzes the conversion of lactate to pyruvate; nicotinamide adenine dinucleotide (NAD) is reduced to NADH in the process (53). In serum, LDH can be separated into five different isoenzymes. Elevated serum levels of LDH have been observed in many medical conditions and diseases. The highest levels are seen in patients with megaloblastic anemia and shock. Moderate increases are seen in muscular disorders, nephritic syndrome and liver cirrhosis while mild increases in LDH activity are seen in myocardial and pulmonary infarction, leukemia, hemolytic anemia and non-viral hepatitis (65).

1.5.2 AST

Aspartate aminotransferase (AST) is an intracellular enzyme found in hepatic, myocardic, muscle and kidney tissue. Diseases in these organs, including hepatobiliary conditions, ischemic heart disease and viral hepatitis lead, to an increase in plasma activity (65, 66). Two iso-enzymes of AST have been reported, one in the cytoplasm and one in the mitochondrial, but only cytoplasmic AST is normally found in plasma. AST transfers an amino group from glutamate to oxaloacetate forming aspartate (53).

1.5.3 ALT

Alanine aminotransferase (ALT) is present mainly in the liver but also in other tissues in very low concentrations. Therefore ALT is considered as a liver specific enzyme and an increased plasma activity is seen in different kinds of hepatic disease. The decline rate is slow in plasma with a half life of 36h (65). Normally ALT catalyzes the reaction where the amino acid alanine loses its amino group by transamination to form pyruvate. Preterm human infants cannot convert alanine to glucose (67) suggesting a low intracellular ALT activity under normal conditions.

1.6 *Pathophysiology of asphyxia: Aspects of specific organs*

1.6.1 The central nervous system

The central nervous system (CNS) is not uniformly sensitive to HI, different areas have different vulnerability. This sensitivity difference depends partly on the degree of maturation. As such a different injury panorama is seen between term and preterm infants. Also, particular types of cells in the brain are more susceptible to HI. For example, glial cells are less vulnerable than neural cells in the newborn brain. During the first week of life, injuries to the basal ganglia, thalamus and cortex are common in term infants after asphyxia but damage to the midbrain, brain stem and hippocampus is also seen (6). Occasional selective injuries of the white matter are reported in term infants (6). Magnetic Resonance Imaging (MRI) gives high resolution images of the brain. Particular pattern of changes on these images are associated certain types of injury to the brain some of which are good predictors of outcome (68) and can even suggest timing of the insult. Such changes are swelling of the brain, increased signal intensity in the cortex, loss of grey-white matter differentiation, abnormal signals in the thalamus and basal ganglia, haemorrhage and focal infarction (6, 69, 70)

The vulnerability to HI and the distribution of injury depends on gestational age (maturation). In the immature brain, it is known that both necrosis and apoptosis, the two modes of cell death, contribute to neurological injury after HI in the neonatal period (71) but the proportion of the two modes of cell death differs with the type of insult. As a rule, severe ischemia results in a lot of necrosis while milder insults cause a more prolonged cell death, mainly by the mechanism of apoptosis. In Myers classical work using a newborn monkey model of asphyxia, (72) four groups of histopathological patterns in the brain were seen depending on the pattern of oxygen deprivation given to the animals (Table 3).

| Oxygen deprivation | Effect |
|---------------------------|---|
| Anoxia | Brainstem damage |
| Hypoxia with acidosis | Cortex damage predominance |
| Hypoxia without acidosis | White matter damage Basal ganglia damage |
| Hypoxia plus anoxia | predominance |

Table 3 Histopathologic effects on the newborn primate brain after four different types of oxygen deprivation. Adapted from Myers (72).

1.6.1.1 Cell death in the brain

Many of the mechanisms involved in neuronal death in the immature brain are the same as those described in section 1.4.1. However, some important differences exist and are these are described in the following section

The brain is a complex, never resting, system with high demands on ATP supply. The most demanding process, using as much as 50% of its metabolism in the CNS, is the maintenance of the ion gradients, mainly the active pumping of sodium cross the cell membrane. Lack of oxygen will, as described in the previous section, cause depletion of ATP followed by less effective Na/K pumps resulting in a decreased ion gradient as the beginning of the cascade of events leading to irreversible or reversible cell damage. Phosphocreatinine [PCr] is a high-energy compound that can donate a phosphate group to ADP to form ATP in a reaction catalyzed by Creatine Kinase (CK) (53). The ATP concentration decreases appreciable when

the PCr/Pi ratio is so low that this reaction cannot replace the ATP fall sufficiently. In newborn infants with severe asphyxia the cerebral energy metabolism, assessed by phosphocreatine concentration [PCr]/inorganic orthophosphate concentration [Pi] ratio, decreases the first days after birth (73). This energy failure occurs in spite of normal mean arterial blood pressure, arterial pO₂, blood glucose and cerebral intracellular pH (73) and is termed “secondary” or “delayed” energy failure because it is preceded by an immediate drop in [PCr]/[Pi] ratio (72). There is a relationship between the severity of the secondary energy failure and poor long term (12 months) prognosis (73, 74). The biphasic energy failure is also illustrated with phosphorus magnetic resonance spectroscopy in newborn pigs in Figure 5. The mean cerebral [PCr]/[Pi] ratio shows an immediate decrease during acute HI followed by a normalization during the first hours of recovery. Thereafter, the [PCr]/[Pi] ratio decreases during the 48h following HI.

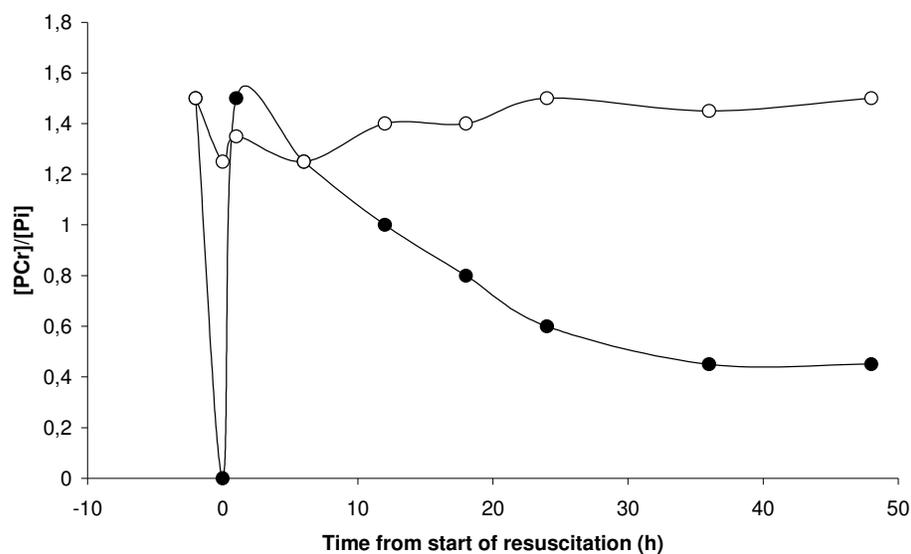


Figure 5. The biphasic cerebral energy failure seen as [PCr]/[Pi] ratio in newborn pigs subjected to hypoxia-ischemia ● compared to controls ○ (Adapted by Lorek et al 1994) (75).

Thoresen (76) describes the cut off levels for ATP depletion where the neurons are damaged.

At a level less than 25% of normal neurons become negatively affected. When ATP levels

drop to less than 10% of normal condition will cause irreversible damage and death of the neurons. Interestingly this cut offs are pretty much the same in different ages and species. The pathophysiological mechanisms of HI injuries specific to the brain that follows the ATP depletion are now described.

1.6.1.2 Excitatory amino acids

Both the pre and post synaptic membranes contain calcium channels where glutamate mediates the signal transduction (77) that activates N-methyl-D-aspartate (NMDA) or α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. When activated, Na^+ will follow its electrochemical gradient into the cell through the AMPA gated channel. This depolarization will remove the Mg^{2+} block of the NMDA channel and finally cause calcium influx into the cell through voltage-sensitive calcium channels. The resulting calcium overload activates proteases, lipases and endonucleases initiating a cascade of events leading to cell death (78).

Excess glutamate release is a central event in the pathophysiology of HI induced cell death and neuroprotection is seen when glutamate is inhibited in animal models (79, 80)

1.6.1.3 Protein synthesis in the brain

After an episode of HI, protein synthesis returns to a level seen before the insult in some regions of the brain while it remains blocked in more sensitive regions. Therefore, a prolonged inhibition of protein synthesis is an indicator of cellular damage post insult. Also, there is a difference in protein synthesis recovery between adult and fetal animals with quicker recovery in the immature brain (81).

1.6.1.4 Free radicals

In the brain, free radicals are produced during reperfusion after HI (82) and pharmacological inhibition of production of radicals, for example, inhibiting of xanthine oxidase by allopurinol, offers neuroprotective effects in animals (83). Similarly, reduction in NO production through inhibition of NO-oxidase has been shown to offer neuroprotection in the immature rat (84).

1.6.1.5 Delayed cell death

One didactic way to describe the pathophysiological events resulting in neurological cell death after HI is in terms of chronologic phases (85). As illustrated in Figure 6, these phases begin with the HI insult. This primary phase includes depletion of ATP, disruption of ion gradients, calcium overload, cell swelling and accumulation of glutamate as described above. Following HI, a “latent” phase with recovery of the energy metabolism (even if the cerebral blood flow still differs from pre-HI levels) begins. This “calm before the storm” precedes the secondary phase of delayed energy failure, which begins as late as 15h after the HI event and is characterized by deterioration in cell integrity and, finally, cell death.

As a clinical symptom, seizures often follow the cellular swelling seen in the secondary phase (17).

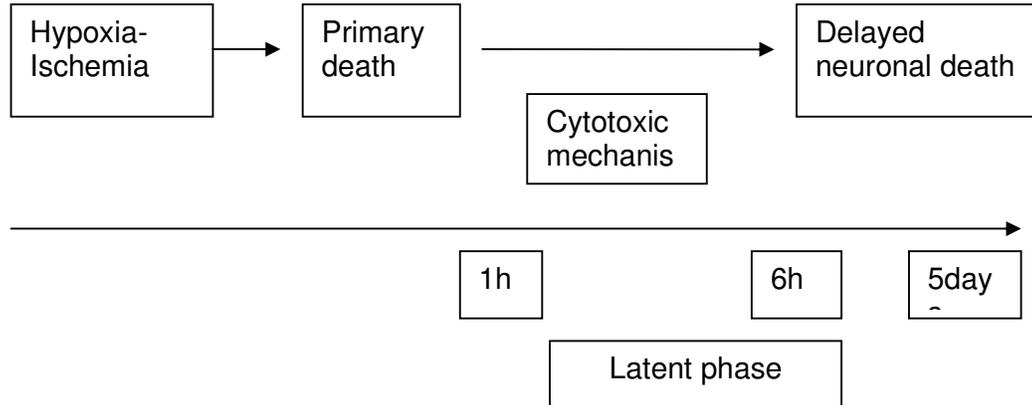


Figure 6 the phases of hypoxia-induced cell death in the immature brain. Adapted from Gunn and Thoresen 2006 (86).

The mechanisms behind the primary and the secondary cell death are not the same. One difference is the absence of intracellular acidosis in the later even if the oxidative phosphorylation is affected (74). Apoptosis may have a central role in the delayed cell death seen in CNS after HI in newborns (87) and there is a relationship between numbers of apoptotic cells and degree of ATP depletion during HI (71). Still, the complete mechanisms of delayed cell death are not known.

1.6.2 Heart/ Cardiovascular response

Severe total or partial asphyxia causes a decreased ventricular contractility and diminished cardiac output (88). This contractile dysfunction of the heart is seen together with biochemical and radiographic evidence of transient myocardial ischemia (TMI) (89-91). In the myocardium, ATP depletion is the main event leading to injury during and after ischemia. After 15 minutes of coronary occlusion the ATP levels in the ischemic tissue are diminished

by 50%. From here they continuously decrease to 22% of baseline over the following three days, before returning to pre-ischemic values by one week (92).

The clinical symptoms of TMI are heterogenic, ranging from tachycardia, murmurs (from tricuspid valve insufficiency), and congestive heart failure to cardiogenic shock in the severe cases (93-95) Due to the insufficient cardiac function hypotension is frequently seen after asphyxia (96, 97). One condition seen in adults and newborns is myocardial stunning (98) causing a persistent but reversible cardiac dysfunction during the reperfusion state due to production of free radicals and insufficient Ca^{2+} homeostasis. Experimental data suggests that the immature heart recovers in functionality better than the adult heart after short periods of ischemia (99) but there are also great differences in the myocardial response to ischemia-reperfusion between newborn and older neonates and between different animal models (100); some of which are now described.

Under normal conditions, the immature heart is less compliant with less preload reserve than the adult heart. The reason for this is structural differences with higher water and protein content in the fetal myocardial muscle. This means that the immature heart is working closer to the peak of the Frank-Starling curve (101). It also works under much higher adrenergic stimulation with a reduced inotropic reserve.

The neonatal heart relies to a greater extent on its own glycogen storage by converting it to glucose through glycogenolysis (102). In the myocardium, calcium activate the proteins thus leading to contraction. The calcium is mainly released from the sarcoplasmic reticulum in the mature heart (103). This is not seen in newborn rats and it seems likely that newborns are more dependent on extracellular calcium compared to adults (103) . This is also seen in other,

more primitive species. Inhibition of calcium channels in the neonatal heart depresses the cardiac function more than in adults (104). Thus, for the immature heart, specific and normal functions such as substantial glycogen stores, improved anaerobic metabolism (102), better calcium exchange during ischemia (105), more ATP due to lower 5' nucleotidase activity (106) and increased amino acid substrate utilization (99) make it less sensitive to ischemia compared to the adult heart. In both neonates and the adult, determination of troponine t (the subunit of the troponine protein complexes that is specific for the myocardial muscle) seems to be a better marker of myocardial ischemia (107) than aminotransferases and creatine kinases (107, 108).

Histological findings relating to the heart reported in neonates after asphyxia include congestion, myocardial and subendocardial hemorrhage or necrosis, cardiomyopathy and infarction of the endocardial muscles (including necrosis of the papillary muscle) (109, 110).

1.6.3 Renal dysfunction

Kidney involvement is common after perinatal asphyxia, as a consequence of the redistribution defence mechanism described in. Clinically, oliguria, increased creatinine levels and electrolyte disturbances are seen (111). The clinical symptoms are usually reversible (112). In asphyxiated fetal sheep, some degree of tubular necrosis was seen in all cases while the glomeruli were spared (113). Medullary necrosis and hemorrhage is seen post mortum in newborn infants after asphyxia (114).

1.6.4 Liver

1.6.4.1 Circulation

The circulation of the liver is unique with its dual supply of blood flow arising from both the hepatic artery and the portal vein (115). In the human fetus, the liver receives approximately 80% of all the blood from the umbilical vein while 20% is shunted through the ductus venosus (116). Surprisingly, the nutrient rich blood from the umbilical vein is not equally shared between the right and left liver lobes with a greater supply feeding the left lobe despite its smaller size (117). In the fetal sheep, the proportion of blood shunted through the ductus venosus increases during hypoxia (118). This alteration in the proportion of blood flow passing through the ductus venosus as compared to the liver is probably dependent on sphincter mechanisms (115).

In fetal lambs during late gestation both oxygen and glucose requirements have been described before and during HI (119). Before HI, the liver accounts for 20% of the total fetal oxygen consumption. During hypoxia, uptake of oxygen decreases by 45% in the right liver lobe while it remains unchanged in the left lobe. Both lobes begin to releasing glucose during the hypoxic episode as a contribution to the anaerobic metabolism (119). The differences in blood flow between the left and the right liver lobe is fascinating, and raises the question why this is happening. Perhaps the answer can be found in the increased capacity of the left lobe for glucose production during hypoxia compared to the right lobe (9mg/min/100g vs. 6mg/min/100g) (119) that could be used as substrate for energy production.

1.6.4.2 The Lobule

The lobule is the basic anatomical unit of the liver (Figure 7). Blood enters the periphery of the lobule via the hepatic artery and portal vein. It then flows via the specialized capillaries of the liver (the sinusoids) and drains into the central vein in the middle of the lobule (120). The periphery of the lobule is called zone 1, the centrilobular area zone III and the middle part of the lobule zone II. Based on the enzyme distribution in the lobule, hepatocytes in zone 1 seem to be specialized for gluconeogenesis while zone III hepatocytes are adapted for glycolysis (121). Various substances such as metabolic substrates, waste products and hormones are removed or added to blood during its passage through the lobule. Oxygen is extracted during its passage from zone 1, through the lobule to the zone 3 creating an oxygen gradient of 40-60 torr (122).

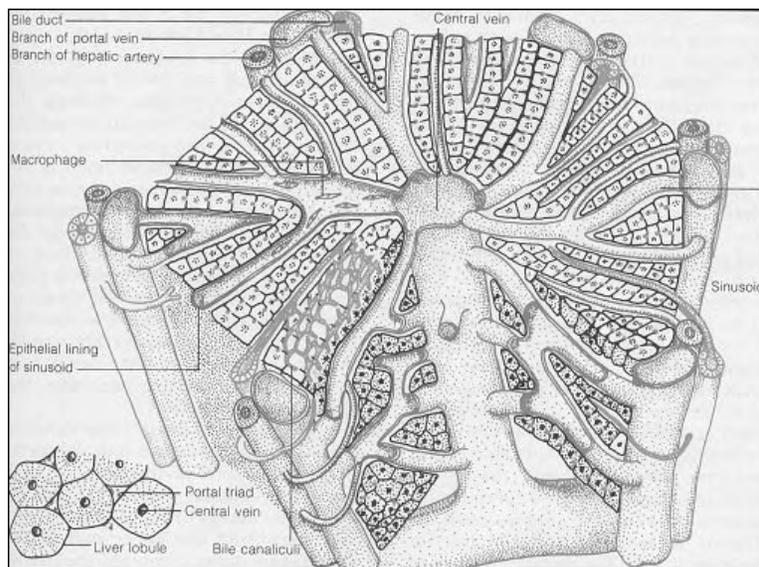


Figure 7 the lobule, the basic anatomical unit of the liver

1.6.4.3 Shock liver syndrome

Liver cell injury commonly occurs after HI, hypotension and poor perfusion (123, 124) e.g. after perinatal asphyxia (2, 44). The terms shock liver syndrome and ischemic or hypoxic

hepatitis are all used for the same condition. It is defined as an early, sharp and transient

increase in aminotransferases (AST and ALT) and LDH plasma activity seen after an acute cardiac or circulatory failure in the absence of viral hepatitis (13). The time course of the enzyme activity pattern in hypoxic hepatitis is similar between patients, with a slightly increased serum AST and/or serum ALT close to the triggering event, followed by a peak concentration of aminotransferases and LDH in serum within 24 to 72h after the insult (9-11). After the peak, aminotransferase levels return to near normal within ten days (13, 14). The prognosis of hypoxic hepatitis itself is good and it rarely progresses into complete hepatic failure (110, 125).

1.6.4.4 HI induced cell death in the liver

The cell death mechanisms, described in section 1.4.1, with mitochondrial dysfunction followed by cellular ATP depletion are largely the same for hypoxic cellular injury in the liver (126). However, even if the mechanisms are similar within the cell there are some differences between the cells during HI. During total anoxia, the zone III hepatocytes are more frequently injured compared to cells in zone I-II (13). This histological finding (centrolobular necrosis) is probably explained by the high oxygen extraction across the lobules which results in that hepatocytes furthest from the oxygen supply are more frequently injured during HI. Hepatocytes are more sensitive to low-flow hypoxia than total anoxia. This is demonstrated in vitro where hypoxic zone II hepatocytes lose viability faster than totally anoxic zone III hepatocytes. This is probably a consequence of the formation of free radicals (127).

There are also some intercellular differences in sensitivity to hypoxia in the liver. Kupffer cells, sinusoidal endothelial cells and biliary epithelial cells are less sensitive to ischemia and reoxygenation injury than hepatocytes during normothermic conditions (126).

The Kupffer cells are the macrophages of the liver and are thus of great importance as the liver is a highly potent immunological organ containing 80% of the all macrophages in the human body (128). This makes the liver a primary site for the synthesis of many acute-phase proteins, cytokines and complement factors (129, 130). Large amounts of inflammatory cytokines, in particular interleukin 1 (IL-1), IL-6 and tumour necrosis factor alpha (TNF α), are present in the normal liver. Their role is to mobilize local and systemic inflammatory responses (130, 131).

Compromised blood flow to the liver, followed by resumption of normal blood flow, causes ischemia/reperfusion (i/r) hepatic injury (132). This injury consists of two phases. The early phase (1-3 hours after reperfusion) involves with free radical generation and activation of the Kupffer cells resulting in hepatocellular injury. The Kupffer cells release proinflammatory cytokines (IL-1, IL-6 and TNF α) (133) that can stimulate the release of neutrophil chemoattractants from liver cells (134). These events in the early phase culminate in the late phase (6-24 hours after reperfusion) with the characteristic accumulation of neutrophils in the liver (135, 136).

The release of pro-inflammatory cytokines seen in i/r injury also seems to have diverse extra hepatic effects (137). Increased circulating concentrations of these cytokines may lead to local cytokine production which in turn leads to hypotension, lung injury and cerebral edema (138). Studies using a rat model support the hypothesis that pulmonary neutrophil chemoattractants, produced in response to hepatic-derived TNF α after i/r injury in the liver, is an important mediator of lung injury (137, 139). Data suggest that pro-inflammatory cytokines may cross the blood-brain barrier and cause direct damage to fetal white matter (140). Cytokines may weaken the blood-brain barrier or the barrier itself may be inefficient if immature (141).

We did a small experiment (n=6) in our newborn pig model of global hypoxia ischemia (presented in chapter 3) supporting an inter-organic relation regarding proinflammatory cytokines. TNF α and IL-6 were analysed before and at fixed timepoints after 45min of global hypoxia ischemia. Blood were collected simultaneously from the umbilical artery (UA), the inferior caval vein (ICV), via a catheter with the tip located at vena hepatica and from sinus sagitalis. As seen in figure 8 there is a trend of increased TNF α plasma values in animals subjected to HI (n=4) both compared to baseline values (before the HI) and compared to the control animals (n=2). Also, the highest mean TNF α value was seen in post-hepatic blood (ICV) with a decrease seen in blood collected in sinus sagitalis (after the brain). The lowest TNF α value was seen in arterial blood. Interestingly these data suggest that TNF α is produced in the liver, transported to the brain where some of the TNF α disappears. These data obviously have to be confirmed in bigger studies but if the liver is shown to produce proinflammatory cytokines with effects in the brain this opens up for new treatment strategies.

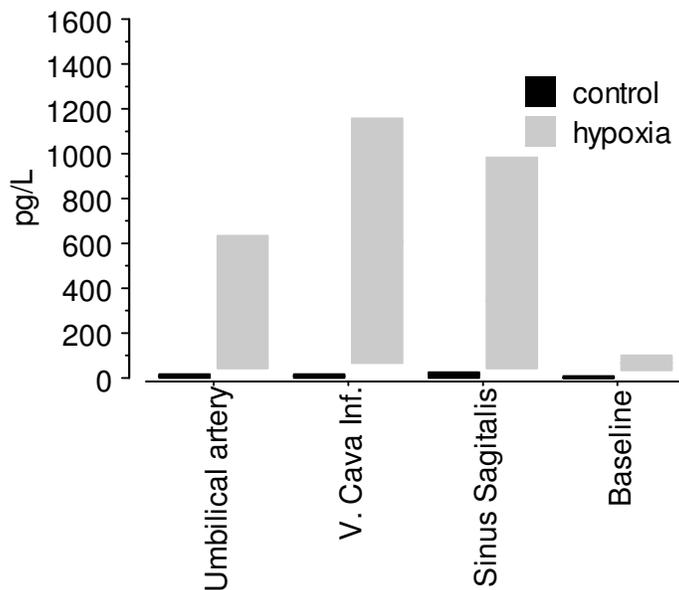


Figure 8 Unpublished data (Karlsson, Tooley, Satas and Thoresen) showing TNF α in different compartments one hour after 45min of global HI compared to baseline value in umbilical artery.

1.7 Hypothermia treatment

The biphasic pattern of HI induced damage of the perinatal nervous system opens up a window of opportunity to minimize damage by intervening before the second phase begins. The medical care of the asphyxiated newborn has, until recently, focused on the secondary events including anticonvulsive drugs, regulation of hemodynamic parameters together with electrolyte and glucose correction. The most promising treatment strategy that is actually focusing on the pathophysiological events leading to HIE is hypothermia (HT) treatment. Both clinical trials (18, 19, 142-146) and meta analyses of them (147) support that HT treatment is beneficial to term infants with HIE. While induced HT is applied in other medical fields and in patient categories of different ages, this section will focus on its efficacy following global HI in the perinatal period.

1.7.1 History

In the late 1950s, Bernard and colleagues (148) described the spontaneous decrease in temperature of 2°C seen in asphyxiated newborns as an indication of endogenous cooling mechanism after HI. Prior to this, experimental data from different animal models showed that hypothermia induction during anoxia was neuroprotective (149, 150). A protective effect of HT treatment *after* the insult in the neonatal period was also described in several studies during the 1950s and 1960s. This early work is well summarized in a review by Thoresen from 2000 (76). Studies showing serious side effects in pre term babies exposed to HT (151) resulted in HT treatment gaining a bad reputation for a period and the method was not investigated further for many years. A new dawn for HT therapy was seen two decades later when mild hypothermia, defined as 32-35°C was proven to be neuroprotective in animals (152).

1.7.2 Hypothermia and the brain

HT following HI is neuroprotective in experimental models across different species (15-17), with both short and long term survival (153, 154) and after a delay between the insult and treatment (155, 156).

The mechanisms by which hypothermia offers neuroprotection are only partly understood and hypothermia have been shown to inhibit many of the mechanisms involved in delayed neuronal death. As described previously, if the oxygen and glucose supply is diminished due to insufficient gas/blood exchange between placenta/lungs and the fetus/newborn, the blood flow to less important organs will decrease. This will cause a need for the organism to minimize the metabolic demands. One way of doing this is by lowering body temperature. In newborn pigs, the metabolism in the brain decreased more than 5% for every 1°C decrease in temperature (157) delaying the onset of anoxic cell depolarization and in a paper from Thoresen et al (Ped. Res. 1995) (158) the brains from newborn pigs showing amelioration of secondary energy failure during hypothermia (Figure 9).

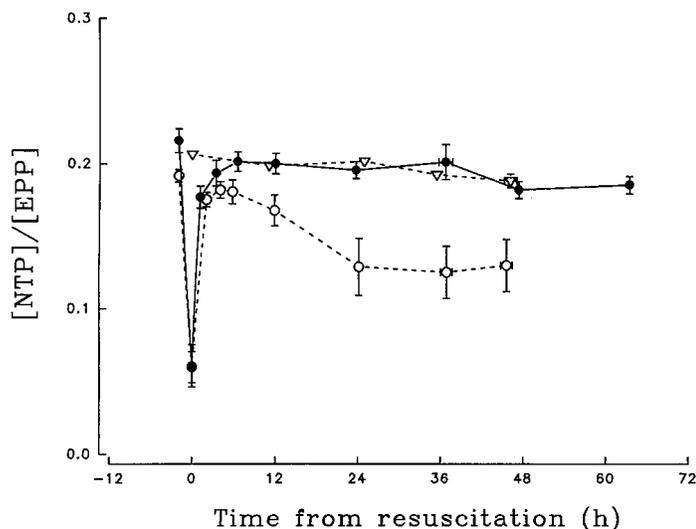


Figure 9 Nucleotide triphosphate/exchangeable phosphate pool ratio [NTP]/[EPP] determined by phosphorus magnetic resonance in three groups of newborn pigs ●= hypothermia, ○= normothermia and ◇= sham operated controls (158).

Hypothermia lowers the release and increases the uptake of glutamate (159) and inhibits the production of free radicals (160, 161) There are also studies suggesting that hypothermia suppresses the proinflammatory response seen after HI e.g. in terms of suppressed proinflammatory cytokine release.

1.7.3 Hypothermia and the rest of the body

Both clinical and experimental studies show that systemic HT has physiological effects on most of the body's organ systems. Whether these effects are beneficial or adverse depends on study design, age and degree of HT. Some of these effects could result in side effects of clinical relevance, particularly with extended or deep HT, but newborn mammals, including human infants, do not seem to be vulnerable to mild HT. There is broad evidence of the positive effects of HT on organ pathology in older animals (162, 163).

The adverse and positive effects of HT on the extra-cerebral organs are reviewed below.

1.7.3.1 Cardiovascular effects

Cardiac output (CO) is decreased during mild HT. This decrease in CO will partly be compensated by increased systemic vascular resistance with a decreased blood flow to different organ systems including the heart, kidneys, gastrointestinal tract (164) and lungs (165). At the same time, the oxygen is bound tighter to the hemoglobin. This shift of the oxygen dissociation curve to the left is partly due to reduction in pCO₂ that accompanies HT (166). This combination of decreased CO, increased vascular resistance and less oxygen delivery to the tissue due to left shifted oxygen dissociation curve (that is already to the left in newborn because of the fetal haemoglobin) means that HT could potentially be dangerous to

already hypoxic peripheral organs. In addition, in terms of perfusion of these organs, low temperature correlates with increased blood viscosity, fibrinogen and haematocrit (Hct) (167, 168) resulting in a potential decreased organ perfusion. However in a Doppler echocardiography study on newborn pig no association between cooling and; pulmonary artery pressure; presence of persistent ductus arteriosus; mean arterial blood pressure or heart stroke volume was seen. When subgrouped, hypothermic pigs subjected to a severe HI insult showed a reduction in cardiac output due to decreased heart rate (169)

1.7.3.2 Hematology

The coagulation cascade is catalyzed by enzymatic reactions that are less effective during HT (170) increasing the risk for coagulopathy. The trombocytes are affected by HT both in a decrease in the numbers of circulating trombocytes (171, 172) and reducing their ability to aggregate (173). Experimental studies describe an impairment in neutrophil migration and bacterial phagocytosis by HT (174-176). It is theoretically possible that this could make the patient more susceptible to infections. Mild HT in near-drowning patients and during colon surgery increases the incidence of clinical infections (177-179). However, this is not the case in adults subjected to 24h of HT (32-33°C) after traumatic brain injuries (180).

1.7.3.3 Renal function

HT, even mild, induces diuresis due to increased renal blood flow, decreased reabsorption in the distal tubuli and also decreases the effect of vasopressin (181). The reduced reabsorption also includes electrolytes with an increase, in sodium excretion reported (182).

1.7.3.4 Liver

Liver functions, including detoxification of ammonia, are well preserved during mild HT (183) and even when the liver is cooled to 4°C before organ transplantation. In dogs subjected

to hepatic vascular occlusion for one hour at normothermia (NT), the liver was severely damaged and all dogs died. Dogs cooled to 25-31°C had 100% survival and an absence of liver damage (162). HT (29°C) in young pigs undergoing cardiopulmonary bypass surgery has been shown to result in less liver necrosis (163). A decrease in pathological findings in the liver in pigs subjected to interruption of blood flow to a liver lobe has been seen when rectal temperature was lowered to 33-35°C before interruption (184). No experimental studies support the hypothesis that induced mild HT after HI in the neonatal period has positive effects on liver injuries. However, a smaller clinical study shows a reduction in aminotransferases is seen together with HT treatment in asphyxiated newborn 72h after birth (185).

As in the brain, the mechanism of the protective effects of HT on the liver is, like in the brain, not fully understood. However, a decrease in metabolic demands is one of the explanations (186) also in addition to inhibition of proinflammatory cytokines (187) and free radical production (188) in the reperfusion phase. HT seems to have effects on both necrotic and apoptotic cell death in the liver (189).

1.7.4 The four questions about hypothermia treatment

The three main questions to be answered when it comes to HT treatment of HIE in neonates after asphyxia are:

How cold?

How long?

How late?

Is it dangerous?

These questions are not easy to answer considering that the differences in systemic response to HI and brain maturity between the animal models used for research. In addition, inter-individual differences and the short and long-term perspective of the neuroprotective effects must be considered. Following experimental studies, treatment should undergo clinical trials. However, the low incidence of HIE together with the mix of ante- and intrapartum asphyxia further complicates the matter.

Previous experimental and clinical studies investigating neuroprotective effects of induced HT in the neonatal period are summarized in Table 4. Inclusion criteria in this meta analysis were; the paper must be written in English and published after 1989, HT should have been induced after the HI insult, with the purpose to investigate effects on the CNS neuroprotection after perinatal asphyxia or HI. The search was performed on Medline, with the term “Hypothermia” limited to “newborn”. All case reports and reviews were excluded.

| Species | Delay (h) | Temperature °C (reduction) | Duration (h) | Neuroprotection (short/long term) | Type of protection |
|------------------------------|-----------|----------------------------|--------------|-----------------------------------|----------------------|
| Rat (p7) (190) | 0 | 32.5 (15%) | 3 | Yes (short) | Histological |
| Sheep (fetal, preterm) (191) | 1.5 | 29.5 (25%) | 68.5 | Yes (short) | Histological |
| Humans (term)(192) | <6 | 34-35 (5-8%) | 70 | Yes (short) | Head CT scan |
| Human (term) (193) | | | | | |
| Human (term) (194) | <6 | 33.5 (9%) | 72 | Yes (long) | Death and disability |
| Human (term) (195) | <6 | 33-34 (8-11%) | 48-72 | Yes (short) | MRI findings |
| Human (term) (18) | <6 | 34-35 (5-8%) | 72 | Yes (long) | Death and disability |
| Human (term) (142) | <6 | 33 (11%) | 48 | Yes (long) | Death and disability |
| Human (term) (145) | <6 | 33-34 (8-11%) | 72 | Yes (short) | MRI findings |
| Human (term) | <6 | 32-34 (8-14%) | 72 | Yes (long) | Disability and MRI |

| | | | | | |
|--------------------------|-----|------------------|-----|----------------------|--|
| (196) | | | | | findings |
| Rat (p7) (197) | 0 | 30 (19%) | 24 | Yes (short) | Decreased Caspase-3-like activity |
| Humans (term) (144) | <6 | 34.5-36.5 (1-7%) | 72 | Tendency (long) | Disability |
| Humans (term) (143) | <6 | 36-36.5 (1-3%) | 72 | Tendency (short) | EEG abnormalities and PAF concentration |
| Piglet (Sedated) (198) | 0 | 34.9 (9%) | 12 | Yes (short) | Apoptotic cells |
| Piglet (Unsedated) (199) | 0 | 35 (10%) | 24 | No effect | No effect |
| Piglet (Sedated) (16) | 0 | 34-35 (10-13%) | 24 | Yes (short) | Histology and neurology score |
| Piglet (Sedated) (15) | 0 | 35 (10%) | 12 | Yes (short) | Secondary energy failure |
| Sheep (fetal) (17) | 1.5 | 32.4 (17%) | 72 | Yes (short) | Secondary cortical oedema EEG Infarction/neuronal loss |
| Rat (p7) (153) | 0 | 32 (14%) | 6 | Yes (long) | Histopathology |
| Rat (p7) (200) | 1.5 | 32 (14%) | 3 | Yes (long) | Performance and histopathology |
| Rat (p14) (156) | 0-6 | 30 or 33 (10-18) | 2-6 | Yes (short) 6-12h | Cerebral energy metabolism Histopathology |

Table 4 Original research articles between 1990 and 2008 investigating neuroprotective effects from induced hypothermia after hypoxia-ischemia in the perinatal period

1.7.5 Modes of cooling

The two different modes of cooling that have been evaluated in clinical trials are selective head cooling (SHC) and whole body cooling (WBC). SHC is induced by a cooling cap (Olympic Medical, Seattle US) that looks like a helmet with circulating cold water (Figure 10). WBC may, for example, be induced by positioning the infant on a cooling blanket (Tecotherm TS 200 Tec, Com Halle Germany). It has been suggested that SHC is superior to WBC, offering greater brain protection coupled with fewer adverse systemic effects related to poor perfusion, as a warmer body temperature is maintained. It is not possible to cool the adult brain using SHC (201). Mathematic models suggest that this will not be possible in the newborn either despite the proportional size of the neonatal head differing significantly from

that of the adult (202). This model has been proven untrue in newborn piglet where SHC induced with a cooling cap is an effective method (203).



Figure 10 Hypothermia treatment applied as selective head cooling induced by a cooling cap (Olympic Medical, Seattle US) and whole body cooling (Tecotherm TS 200 Tec, Com Halle Germany) in newborn infants

1.7.6 Clinical trials

Recent, multicenter, randomized controlled clinical trials have used two cooling methods; SHC combined with mild systemic hypothermia to the rectal temperature (T_{rectal}) 34.5°C (18) or WBC to T_{rectal} 33.5°C (19, 20). In newborn, term infants with hypoxic ischemic encephalopathy (HIE), cooling by either method improved neurodevelopmental outcome (204). The two larger multicenter trials of HT applied to neonates (18, 19) did not aim to examine organ protection after HT, however, some cardiovascular and biochemical results were reported and no differences between cooled and non-cooled infants were seen. In a smaller WBC trial by Eicher and colleagues (20) where less mature infants (≥ 35 weeks gestational age) were also included and the most profound cooling (33.0°C) of the three clinical trials was applied, the cooled infants needed longer inotropic support than the normothermic infants. Clotting times in the cooled infants were also abnormal more often.

1.8 *Prediction after hypoxia ischemia*

1.8.1 Why prediction

When a child is born with perinatal asphyxia both parents and medical staff, wants to know how bad the child is injured. The parents need adequate information about what will happen to their child, the physician need information when counselling the parents and for decisions regard withdrawal of medical support. In developing countries where limited resources forces the health care system to prioritize which patient should be offered medical care, the need for rapid and cheap predictive markers are needed.

Clinical trials suggest long term benefits of mild HT treatment in infants with moderate to severe HIE (18, 19, 142), when given as soon as possible after the insult. A prognostic tool early after birth would make it possible to select candidates for HT treatment and thus target available treatment most effectively.

1.8.2 Methods of prediction

There are several neurophysiologic (conventional 12 lead EEG, cerebral function monitoring, visual and somatosensory evoked potentials), biochemical (proinflammatory cytokines, blood gases, neurospecific proteins, nucleated red blood cells and lactate/creatinine ratio in urine), clinical (Apgar score, duration of resuscitation, HIE classification etc.) and radiological (ultrasonography, magnetic resonance imaging and computed tomography) techniques studied for prediction purposes after asphyxia in neonates. In this thesis only a few methods, which have relevance to our studies, will be described.

1.8.2.1 Cord blood gas

The incidence of seizures and neonatal mortality is increased in infants with an umbilical artery pH <7.0 directly after birth (205). But even below this very low pH level, the specificity is low with normal outcome reported in as many as 80% of infants with an umbilical artery pH <7.0 (95). Acidosis is usually both respiratory and metabolic (205). There is an interesting study showing a high predictive value of arteriovenous pCO₂ difference for identification of HIE after asphyxia (206). A limitation of the usefulness of cord blood gas as a predictor is illustrated in the newborn monkey where total asphyxia causes severe acidosis together with a decrease in pO₂ and hypercapnia, while pH and pCO₂ may remain normal during partial asphyxia where the respiratory gas exchange to the fetus is insufficient for a longer time and diminishes gradually. This partial “non-acidotic” asphyxia still causes low pO₂ and is followed by white matter injury (72).

1.8.2.2 Amplitude Integrated EEG

Amplitude integrated EEG (aEEG) also known as cerebral function monitoring (CFM), is used for bedside detection of seizures or depressed background activity. The aEEG output is a very time compressed (1 hour is printed as a 6cm trace), filtered and transformed single channel EEG signal. For comparison raw EEG is run at 30mm/sec hence these outputs are not directly comparable. Studies show that aEEG is a good predictive method for adverse neurological outcome within the first 12h after birth. Hellstrom-Westas et.al (207) showed that aEEG tracing during the first 6h post partum predicted outcome correctly in 43 out of 47 (91.5%) asphyxiated infants, a results that has been confirmed by others (208).

1.8.2.3 Lactate

Anaerobic metabolism during hypoxia leads to production of lactate from pyruvate, a reaction catalyzed by the enzyme LDH. Retrospective data shows that plasma lactate concentration over 7.5mmol/l within 60 minutes after birth is associated with moderate or severe HIE with a sensitivity of 94% and specificity of 67%. Both sensitivity and negative predictive value were better than for pH or base excess (209). However, there is always a steady state between pyruvate and lactate, meaning that an increase in glucose also results in a lactate increase even under normoxic conditions (e.g. glucose infusion, and catecholamines) (39). In cord blood, the predictive value of lactate for neurological abnormalities is similar to pH and base excess (sensitivity 67%, specificity 74%) (210) and as a marker of 12 months neurodevelopmental outcome the sensitivity is poor (12%) (211).

1.8.3 The predictive value of intracellular enzymes

The leakage of intracellular components described in previous sections makes it possible to use some components which are easy to measure in plasma (e.g. LDH, ALT and AST) as biochemical markers of cellular damage after HI. Even if these enzymes have been in use for many decades, the predictive value of LDH, ALT and AST in term newborns with asphyxia is not thoroughly investigated. Lackmann et.al (12) investigated whether LDH and AST during first 72h post partum could predict HIE or periventricular-intraventricular hemorrhage in term and preterm babies. In their study, 10 out of 49 full term infants developed HIE and the sensitivity and specificity for the analyzed enzymes for prediction of HIE were 90% and 71% respectively.

Data on the long term prognostic value of LDH, ALT and AST in infants with HIE are presented in a previous study by Yoneda (212). However, the aim of the study was to investigate the predictive value of serum calcium for long term poor outcome in newborn

infants with HIE. Sensitivity and specificity for LDH and ALT for prediction of abnormal outcome were approximately 80% while AST was less sensitive (73%) with a specificity of 88%.

1.9 *The newborn pig model*

1.9.1 Newborn pigs and human infants

1.9.1.1 The organ systems

The pig pregnancy lasts 115 days as compared to the rat which is 21, sheep 149, chimps 227, human 280 and the Asian elephant which is 645 days. On developing an animal model that can mimic human condition one would like to study, the most important factor is to match the degree of maturation and physiological responses to induced changes. The pig at term is the only large animal where the brain has its peak maturation around the time of birth. For comparison, the sheep peaks well before birth, just like the monkey and the guinea pig. The rat brain peaks around postnatal day 7-8. Hence examples of term equivalence in brain development in other species would be prenatally in fetal lambs and postnatal rat (213). This peak in brain maturation in the pig is also reflected in that its largest brain to body weight ratio is at birth. The newborn pig's brain is 2.0 % of body weight at birth (214) and < 0.5 % in adulthood. No species has a larger brain proportional to body size at term than the human (approximately 10% of total body weight). The term pig has similar brain maturity to term human infants. Neuronal proliferation peaks in the last 5-6 weeks of fetal life and continues for another five weeks after birth for both in pigs and humans (213). The myelination of the brain is more advanced at birth in the pig than the baby and (215) where this process continues long after the neonatal period (216).

The major arterial anatomy of cerebral vessels in pigs is similar to humans with the exception of a network of collaterals between intra and extra-cerebral arteries in the pig the “rete mirabilis” (217). The circulatory response to changing levels of carbon oxide (CO₂ reactivity) and blood pressure (cerebral autoregulation) is also similar to newborn infants. (218).

With regards to the liver a substantial anatomical difference between pigs and humans exists. The pig liver has four lobes and the human two (figure 11). In terms of function, the pig liver has a similar maturity in hepatic metabolism and biotransformation of drugs as human infants (219-223), However under normal conditions the responsiveness of some enzymes relevant to liver function are relatively low at birth in the pig compared to the term infant (220, 223). The inability of the liver cells to use alanine as a substrate for glucose production is seen in both newborn term pigs and pre term human babies suggesting a low intracellular ALT activity in both (224) (67). This difference in alanine metabolism is seen even if the substrate is given exogenously (224) which suggests that a lack of enzymes needed for the reaction is the rate limiting factor.

Under normal conditions healthy newborn pigs and human infants show a similar pattern of enzyme plasma activity after birth with a substantial increase in AST and LDH during the first 12-24h after birth followed by a decrease, returning to baseline levels at 72h of age (225, 226). Also, the absolute plasma levels of AST, ALT and LDH are similar between newborn pigs, term and preterm human infants (with a mean (\pm SD) gestational age of 35 \pm 1.9 weeks) during first three days of life (9-12, 227).

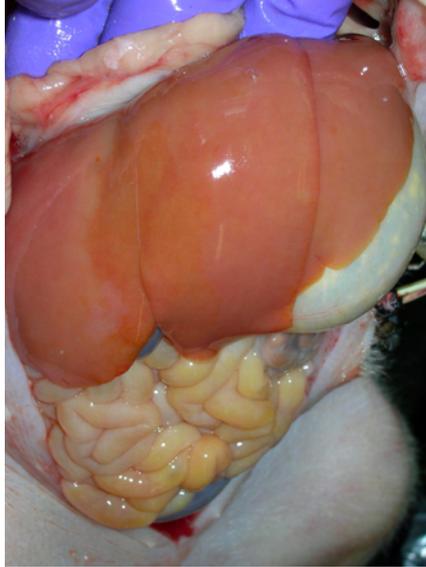


Figure 11 Normal pig liver in anterior view showing the four lobes.

As in the guinea-pig, the pig does not have an anatomical ductus venosus (228). However, under normal conditions, more than 50% of the blood flow in the umbilical vein bypasses the liver in the fetal pig (229) via vascular channels that appear to have the same function as the ductus venosus.

The development of glomeruli in the human kidney is completed 34 weeks after conception followed by an increase in glomeruli filtration rate (230). This is a fixed time point independent of the gestational age when the child is born. The glomerulogenesis in pigs on the other hand is not completed until third week of extrauterine life (231)

One important similarity to newborn infants is the size of the newborn pig which makes it possible to monitor vital function and administrate fluids and drugs through lines and umbilical catheters. The blood volume of approximately 100ml/kg also makes it possible to perform repeated blood sampling (up to 5ml/kg/24h) without affecting the physiology. There is however an important difference between humans and pigs regards the blood- pigs do not

have fetal hemoglobin and the normal hemoglobin value at birth is ~8g/100ml. Lactate levels are shown to increase when anemia is clinically important hence this can be used to ensure sufficient hemoglobin and O₂ transport capacity (232)

Conventional human tests such as the prothrombin time and activated partial thromboplastin time can be used in many mammalian species including all common laboratory and domestic animals. There have been previous studies of the effect of temperature on coagulation times in adult pigs but no studies using neonatal blood. The neonatal coagulation system is also different from the adult system. Pro-coagulant factors 2, 7, 9 and 10 are significantly reduced in newborn blood as are some of the anticoagulant proteins. In a pilot study from the Thoresen laboratory (233) APPT levels were examined at 39°C (which is normal temperature for newborn pigs) and 29°C. The increased APPT was moderate and without clinical significance as haemorrhage was never seen during hypothermia.

1.9.1.2 Hypoxia Ischemia in the newborn pig

Most piglet models used in HI research produce an insult largely confined to the brain (75) using transient bilateral carotid artery vessel occlusion. One model using hypoxia until a certain level of hypotension (20 mmHg) occurs is used for short term acute insults however this model has not been developed into a survival model (234). In the 1990s Professor Thoresen developed a model of global HI in the newborn pig where the whole animal is subjected to HI (235) in which the effects of HI on peripheral organs like heart, liver, kidneys, gut could also be investigated. This model titrate the hypoxic challenge depending on the response seen on the EEG, thus compensating to some extent for the individual tolerance to hypoxia which increases the variability so much in other models. The global HI model mimics the situation seen during and after perinatal asphyxia in most respects and is therefore a model suitable to examine systemic and neurological effects of the insult simultaneously.

One aspect is however different, the CO₂ is kept stable and in the normal range which is unlike the classical asphyxia where CO₂ is high. This was a choice made to minimize the factors that affect brain injury- the levels of CO₂ and relationship to cerebral blood flow.

The newborn pig model of global HI displays the same type of organ injury and cardiovascular responses as seen in the asphyxiated infant (16, 235-237).

The heart of the newborn pig is very vulnerable to hypoxia (234) and it is therefore of importance to individualize the exposure to HI during experiments e.g. by EEG monitoring where the oxygen fraction is regulated to the highest value still giving a low EEG amplitude (<7μV) (235).

Similar types of brain injuries seen in the asphyxiated infant are also seen (16, 235-237) and are distributed to cortex, white matter, basal ganglia, hippocampus, thalamus and the cerebellum. The pig is the only animal model with postnatal survival that develops posthypoxic seizures, another feature typical for the encephalopathic newborn child. The similarities in cardiovascular and systemic responses to HI make this model suitable for research on the pathophysiological mechanisms of HI injuries of the brain and body.

Global HI is known to cause liver damage in the newborn pig with histological findings similar to the injuries seen in term newborn infants (237-240)

2 AIMS OF THE INVESTIGATION

The general purpose was to study the biochemical response to organ damage after perinatal asphyxia and the potential impact of hypothermia treatment on the same.

The specific aims were to study:

- the occurrence of hypoxic hepatitis in full-term infants after birth asphyxia
- the temporal enzyme pattern in asphyxiated newborn infants
- if the degree of hypoxic hepatitis as reflected by the aminotransferase rise correlates with the severity of the asphyxia and CNS symptomatology.
- whether 24h of hypothermia treatment, initiated 3h after global hypoxia ischemia is neuro- or organ-protective in newborn pigs
- whether any differences exist between selective head cooling and whole body cooling regarding organ and brain pathology and biochemical markers in newborn pigs subjected to global hypoxia ischemia.
- whether plasma values of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH), differ between newborn pigs with liver injuries after a severe global HI insult and pigs with normal livers.
- whether LDH, ALT and AST activity during the first 12h after birth predicts HIE in newborn term infants with intra partum signs of fetal distress.

3 MATERIALS & METHODS

3.1 *Ethical considerations*

In Sweden, the clinical studies have been approved by the regional ethics committee (Karolinska Institute, Dnr: 99/395 and 109/02) and oral consent was obtained from all parents. In the UK protocols were approved by the University of Bristol Ethical Review Panel and carried out in accordance with British Home Office guidelines.

3.2 *Patients, samples and design: Clinical studies*

Paper I

Twenty-six asphyxiated full-term infants were prospectively studied. The primary inclusion criteria were an Apgar score < 7 at five minutes after birth and a gestational age of more than 36 weeks. At least two of the following criteria also had to be present: (1) Umbilical cord arterial pH < 7.20, (2) mild, moderate or severe hypoxic ischaemic encephalopathy (HIE), as defined by Sarnat and Sarnat [12], (3) resuscitation with more than three minutes of positive pressure ventilation before stable spontaneous respiration.

None of the included infants had a primary disease of the liver or biliary tract. Infants with bacterial sepsis, and/or potentially hepatotoxic drug-therapy were included.

The control group of healthy newborns (n=56) were defined as healthy when discharged with the sole diagnosis “Neonatal period without complication”. Complete obstetric histories were obtained from the obstetrical records.

In the asphyxiated group, AST, ALT, LDH, gamma glutamyltransferase (GGT), albumin, bilirubin (total and conjugated), cholinesterase activity, prothrombine time (INR) and nucleated red blood cells were monitored on postnatal days one, two and three, and once

between days six and twelve. The data were compared to the control group the same samples were collected once at the age of 24-172 hours by venepuncture when other routine blood sampling was performed.

Hypoxic hepatitis was defined as an AST or ALT elevation exceeding the control mean value + 2 SD with a peak value, compared to first blood sample, seen later than 24 hours after birth, and a subsequent decrease to near normal values within 10 days. The presence of this pattern was explored in the individual asphyxiated infants.

Paper IV

The study population included infants with a gestational age of more than 36 weeks showing signs of intra partum fetal distress (n=301) defined as a non-reassuring fetal heart rate trace (tachycardia, bradycardia, decreased variability, loss of accelerations, late or variable decelerations) and/or blood or meconium stained amniotic fluid which were considered being an indication for operative interventions (caesarean sections, ventouse and forceps deliveries) and/or fetal scalp blood-sampling. The infants were included at the neonatal intensive care unit at Huddinge University Hospital and Sodersjukhuset, Stockholm, Sweden (n=21) at St Michaels Hospital, Bristol, UK (n=26) or at the delivery clinic at Sodersjukhuset (n=254). The profile for inclusion and exclusion in the present study is presented as a flow chart in paper IV.

The plasma activity for LDH, ALT and AST were routinely measured within 12h post partum in the newborn infants with differing degree of HIE (n=44) and in infants where there had been signs of fetal distress but no postnatal clinical signs of HIE they were measured in cord blood (n=202). After stratification based on clinical diagnosis (asphyxia with no/mild HIE (n=11), moderate/severe HIE (n=33), no asphyxia but with other diagnosis (n=35) and control (n=167), all infants were randomised into two groups. The first group (n=123) was used for

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calculation of cut off limits for the enzymes studied and the second group (n=123) were used for calculation of the predictive value of the enzymes for detection of HIE.

There were an overlapping number of 15 included asphyxiated patients between paper I and paper IV

3.3 *Settings, subjects & study design: Animal experiments*

3.3.1.1 Preparation & Anaesthesia

The animals were kept in a calm, heated environment and bottle-fed pig formula (Faramate; Volac Feeds, Royston, UK) *ad libitum*. Anesthesia was induced in a closed perspex box, with halothane (3%), N₂O (67%) and O₂ (30%), followed by endotracheal intubation and mechanical ventilation with maintenance anesthesia. Antibiotics were administered 12 hourly throughout the study period. Pigs with a MABP less than 40mmHg were treated with two boluses of saline followed by inotropic drugs if necessary. Inhalation anesthesia continued until the onset of hypothermia in paper II and ≤ 3h after the insult in paper III when it was replaced by intravenous anesthesia of propofol and fentanyl for 24h. After anesthesia, intramuscular injections of buprenorphine (10-20µg/kg every 12h) were given for analgesia. The pigs were extubated when spontaneous breathing was adequate.

3.3.1.2 The HI insult

The pig was placed in prone position with pads in the axillar and inguinal regions making it possible for the thorax to move freely. Hypoxia was induced by an abrupt FiO₂ reduction to ~5-6% which results in a low amplitude EEG (LA-EEG) <7µV. This degree of HI has been shown to induce permanent brain injury in the newborn pig (235). If bradycardia and/or

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hypotension occurred the FiO_2 was transiently increased until recovery. The duration of HI was 45min followed by reoxygenation with 30% O_2 until TcSaO_2 was $\geq 92\%$ which usually occurred within 5min.

Clinical seizures were defined as rhythmic pathological movements together with electroconvulsive activity on EEG. Electroconvulsive activity was defined as spike or sharp wave activity with an 100% amplitude increase compared to background activity at a regular frequency lasting $> 20\text{sec}$. Post hypoxic clinical or electrical seizures, if they occurred, were treated as required with Phenobarbital 20mg/kg followed by midazolame or lidocaine.

3.3.1.3 Hypothermia (paper II)

Three hours after HI the pigs were randomized to one of three strategies: (NT) ($T_{\text{rectal}} 39.0^\circ\text{C}$) or (WBC) ($T_{\text{rectal}} 34.5^\circ\text{C}$) using a cooling blanket (Tecotherm TS 200 Tec, Com Halle Germany), (SHC) using a cooling cap (Olympic Medical, Seattle US). Temperatures were maintained for 24h. Rewarming at 0.8°C/h to 39°C was started by removing the cooling cap or blanket.

Body temperature was recorded using a rectal probe inserted 6cm from the anal margin. Brain temperature was measured with calibrated microthermocouples (Physitemp Instruments Ltd, Clifton, NJ, US) in the superficial cortex and also at depth of 2.2cm from the brain surface, at the level of the basal ganglia. All temperatures were recorded digitally every minute.

3.3.1.4 Neurological assessment

An 11-item scoring system was applied before the insult and at fixed timepoints after the insult. The scoring system is based on observations of newborn piglets in the experimental situation by Thoresen et al (235). Originally, the system was based on 9 items: (a) normal respiration, without apnea, retractions, or need for oxygen; (b) consciousness; (c) orientation;

(d) ability to walk on all four limbs in one direction without falling; (e) ability to control the forelimbs; (f) ability to control the hindlimbs;(g) maintenance of steady and equal tone in forelimbs and hind limbs; (h) almost continuous activity when awake; (i) absence of pathologic movements. In addition to these items the ability to suck and vocalisation were noticed. Each item was scored as: 2, normal; 1, moderately abnormal or 0, definitely pathologic giving a maximum score of 22 for a totally normal pig

3.3.1.5 Biochemistry

Arterial blood for biochemical and hematological variables including creatinine, sodium (Na), potassium (K), chloride, magnesium (Mg), calcium (not albumin corrected) (Ca), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT), bilirubin, albumin, total protein, white blood cell count, red blood cell count, hemoglobin, hematocrit, platelet count, glucose, lactate and blood gas analysis were collected from an umbilical artery catheter before the insult (baseline) and at fixed timepoints after the insult. The blood was centrifuged at for 10 minutes at room temperature. The separated plasma was then frozen in aliquots in -80 °C until analyzed. The majority of the biochemical analyses listed above were performed at an accredited central clinical laboratory on an Olympus AU 2700® (Olympus, Rungis, France) with the exception of blood gases including lactate which were analysed using iSTAT Portable Clinical Analyzer (Abbott, Illinois, US).

3.3.1.6 Perfusion fixation & Autopsy

Seventy-two hours after the hypoxic-ischemic insult the animals were reanesthetized and intubated. Under deep anesthesia, brains were perfused through the common carotid arteries with 4% formaldehyde. An autopsy was performed immediately with description of

macroscopic pathology and the placement of the umbilical venous and arterial catheters was documented.

3.3.1.7 Histological preparation and examination

From each brain ten coronal sections were examined, sampled at regular, 0.5cm intervals.

Seven regions of the brain were analyzed including: cortical grey matter; white matter; basal ganglia; hippocampus; thalamus; cerebellum and brainstem. The extent of damage for each region was graded with .5-intervals on a 9-step scale from 0.0 to 4.0 (Table 5).

| Grade | area affected (%) | Morphologic changes |
|--------------|--------------------------|--|
| 1 | ≤10% | Individual necrotic neurons, small patchy complete or incomplete infarcts |
| 2 | 20-30% | Partly confluent complete or incomplete infarcts |
| 3 | 40-60% | Larger confluent complete infarcts |
| 4 | >75% | In cortex; total disintegration of the tissue, in thalamus and basal ganglia; large complete infarcts, in hippocampus; neuronal necrosis |

Table 5 Definition of the histopathology grading used for the 9-step neurology score used in paper II and III. Adapted from Thoresen et al 1996 (235)

The livers were immersion fixed in 4% formaldehyde. After fixation the livers were sliced at 0.5cm intervals and tissue was sampled at standard sites from each of the four lobes, processed and embedded in paraffin wax by standard methods. Six µm sections were cut from each site and stained with hematoxylin and eosin for light microscopy. A perinatal pathologist examined the sections. A scoring system for each section, previously described in our model (237), was used, a score of 0 represented no damage and 1 represented pathological changes. Maximum score was 4 with damage in all four sections. Minimum score was 0 with no pathological findings in any of the four sections. Based on the histopathological score the animals in paper III were divided into two groups, those with no liver pathology, hence a score of zero (the normal group) and those with a liver pathology score of 1 to 4 (the pathology group).

3.4 *Biochemical analysis methods*

In this section written information provided by the manufactures about the two methods used for enzyme analysis in the studies (Roche/Hitachi, Roche Diagnostic and Olympus AU 2700, Olympus) have been used as references. Coefficient of variation and analytic range for the two methods are shown in table 6.

The biochemical analyses in the four papers were performed at accredited central clinical laboratories in Stockholm, Sweden and Bristol, UK. In the UK an Olympus AU 2700® (Olympus, Rungis, France) was used while in Sweden the analysis were performed using a full-automatic Modular Analytics (Roche Diagnostics, Germany). In paper IV patients were included in both Stockholm and Bristol and the enzyme analysis were therefore performed on the two different instruments. However the test principles are the same for both instruments as described in section 3.4.1

3.4.1 Test principles

LDH

The reaction: $\text{Lactate} + \text{NAD} \rightarrow \text{Pyruvate} + \text{NADH} + \text{H}^+$ is used for the LDH test. The initial rate of NADH formation is directly proportional to LDH activity. Activity is determined by photometrically measuring the absorbance.

The most important interference is seen in presence of hemolysis and contamination with erythrocytes because of the high concentration of LDH found in red blood cells.

AST

AST catalyzes transamination of aspartate and 2-oxoglutarate forming glutamate and oxaloacetate. Oxaloacetate is reduced to L-malate and NAD^+ by Malate dehydrogenase. The amount of NADH oxidised to NAD^+ is proportional to the AST activity in the sample. Like all aminotransferases AST require the coenzyme pyridoxal phosphate that is a derivate from

vitamin B6 to function. Thus, lack of this vitamin could cause false low AST activity (53).

Therefore pyridoxal phosphate was added to the reaction

ALT

ALT transfers the amino group from alanine to 2-oxoglutarate to form pyruvate and glutamate. Addition of pyridoxal phosphate to the reaction mixture ensures maximum catalytic activity of ALT. Pyruvate enters an LDH catalyzed reaction with NADH to produce lactate and NAD⁺. The amount of NADH oxidised to NAD⁺ is proportional to the ALT activity in the sample and, as like for AST, pyridoxal phosphate is required for the reaction.

| LDH | Modular | Olympus |
|------------------|----------------|----------------|
| Analytic range | 10-2500 U/L | 0-3000 U/L |
| CV low activity | 2.7% | 2.6% |
| CV high activity | 1.2% | 1.6% |
| AST | Modular | Olympus |
| Analytic range | 5-700 U/L* | 0-1000 U/L |
| CV low activity | 3.1% | 1.5% |
| CV high activity | 1.3% | 1.8% |
| ALT | Modular | Olympus |
| Analytic range | 5-700 U/L* | 0-500 U/L |
| CV low activity | 2.9% | 5.4% |
| CV high activity | 3.6% | 1.6% |

Table 6 Analytic range and coefficient of variance (CV) for LDH, AST and ALT analyzed by Hitachi/Roche (Modular) and Olympus AU 2700. *=calculated analytic range 5-7000U/L

3.5 *Statistical methods*

Paper I

Differences in biochemical variables between the groups were analysed with the Mann-Whitney U-test. Spearman's Rho was used as bivariate nonparametric correlation test and a significance level of $p < 0.05$ was used.

Paper II

A linear regression analysis was performed with number of damaged organs or average brain pathology as the dependent variable. A significance value of 0.05 was used when allowing

independent factors to enter the regression. Data were analyzed with nonparametric statistics; Wilcoxon matched pairs test with Bonferroni's correction for multiple comparisons, Mann-Whitney U test and Kruskal Wallis rank test. Nominal data was evaluated using Chi-squared test or Fishers exact test. Areas under the curve (AUC) for continuous data during the cooling period were calculated with the trapezium rule.

Paper III

AUC for AST, ALT and LDH were calculated with the trapezium rule including values obtained before, at the end of the insult and at five fixed time points during the 72h period following the HI insult in the individual pigs. For comparison between the normal and the pathology group Mann-Whitney U test was used. Physiological and biochemical variables at baseline were compared with the different time points after the HI insult using Wilcoxon's paired test for skewed data with Bonferroni's correction for multiple comparison. To assess the correlation between liver injury score and other organ pathology Spearman's Rho was used. Nominal data were analysed with Fisher's Exact Test. A threshold value of $p < 0.05$ was regarded as significant.

Paper IV

Linear regression analysis was performed with LDH, ALT and AST as the dependent variables to find potential relationships between the enzymes and the independent variables. The enzyme activity were compared between the groups with the Kruskal Wallis rank test followed by a non-parametric "post hoc" test with the Mann-Whitney U test which was performed with Bonferroni's correction for multiple comparisons. Nominal data were evaluated using Chi-squared test or Fishers exact test. Finally the groups were randomised into two halves (241). The first randomised half (R-ROC) was used for calculation of best cut off values for LDH, ALT, AST for detection of HIE. The cut off limits were obtained from receiver operating characteristic curves (ROC). These cut off values were then applied on the

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second randomised half (R-Pred) for calculation of the predictive value of LDH, ALT, AST and pH for detection of HIE.

4 RESULTS

Paper I

A Serum-ALT or AST pattern compatible with hypoxic hepatitis was seen in 12 (ALT) and 11 (AST) of the asphyxiated infants respectively. However, in five and eleven (ALT and AST respectively) asphyxiated infants, the highest enzyme activity was seen in the first sample at less than 12 hours after birth.

After 6 days AST and LDH were normalized, while ALT still was increased compared to the control group ($p=0.007$). No correlation between liver parameters and pH, BE, minutes to spontaneous breathing or Apgar at five minutes was seen. A significant correlation between aminotransferase values and grade of HIE was found on:

- day one (ALT: $R=0.47$, $p=0.022$ and AST: $R=0.41$, $p=0.047$)
- day two (ALT: $R=0.53$, $p=0.025$ and AST: $R=0.48$, $p=0.044$)
- day three (ALT: $R=0.81$, $p=0.002$ and AST: $R=0.81$, $p=0.002$)

Numbers of asphyxiated infants with an early and late peak in aminotransferases in relation to an obstetric history suggesting an ante and intrapartum asphyctic event respectively are shown in Table 7

| AST | Early peak | Late peak | Total |
|--------------|------------|-----------|-----------|
| Intrapartum | 1 | 5 | 6 |
| Antepartum | 4 | 1 | 5 |
| Total | 5 | 6 | 11 |

| ALT | Early peak | Late peak | Total |
|--------------|------------|-----------|----------|
| Intrapartum | 1 | 4 | 5 |
| Antepartum | 0 | 4 | 4 |
| Total | 1 | 8 | 9 |

Table 7 Number of asphyxiated infants divided by timepoint for peak plasma activity (early or late) where information about the possible onset of the asphyctic event was available through the maternal record.

Paper II

There was no difference in average brain pathology score across the three groups or the 12-item neurology score 48h HI across the groups. Average brain pathology and neurology correlated strongly at this timepoint ($r=0.5$, $p<0.001$). There was no significant difference in the distribution of brain injury across the groups.

Pooling the data for regression analysis demonstrated a significant relationship between average brain pathology score and the independent variables of arterial pH at end of insult and duration of seizures.

Significantly fewer damaged organs were observed in males than females (Mean (\pm SD) 1.4 (\pm 1.0) vs. 2.3 (\pm 1.1), $p=0.004$) and there was a significant relationship between the three independent variables; pH and lactate at 3h after the insult (immediately before delayed cooling started) and gender, and the dependent variable, number of organs with pathological lesions. The most frequently damaged organ was the liver ($n=29/50$ (58%)).

Mean (\pm SD) plasma lactate rose to 19.5mmol/L (\pm 3.7) by the end of the insult. The recovery time to normal values after reoxygenation was similar across the groups (Figure 2) with normalization in lactate at 3h post-insult compared to baseline ($p=0.417$). However hypothermic animals normalized their plasma-calcium, magnesium and potassium significantly faster than normothermic ones.

Paper III

Twelve animals showed histologically verified liver injuries with a median (interquartile range, (IQR)) liver pathology score of 3.5 (1.5-4). In all seven pigs were included in the

normal group after histological examination.

At autopsy the tip of the umbilical vein catheter (UVC) was found advanced into the liver in four pigs and outside the liver in nine. The localization was not recorded in five animals and one pig did not get an UVC. The median liver pathology score in the nine pigs with the UVC located outside the liver was 0.5 (0.0-2.0) vs. 4.0 (2.5-4.0) in the four pigs where the tip of the catheter was found advanced into the liver ($p=0.040$).

LDH was significantly increased in the pathology group at the end of the 45min HI insult (928 U/L (567-1031)) compared to baseline (679 U/L (548-866), $p=0.010$). However, this was only transient with no significant difference in LDH from 6h post insult. No significant differences between the pathology group and the normal group in AUC for AST (48 (44-61) vs. 51 (48-74), $p=0.495$), ALT (33 (23-40) vs. 23 (21-28), $p=0.079$) or LDH (773 (574-997) vs. 873 (630-1165), $p=0.497$) were seen.

Paper IV

A significant relationship between the independent variables Apgar score at 5 minutes and degree of HIE and the dependent variable LDH activity was seen in the linear regression analysis. Significant relationships between Apgar score at 5 minutes and ALT respectively degree of HIE and AST were also seen. No other clinical variable showed any significant relationship with the enzymes.

The LDH, ALT and AST activities were significantly higher in both the asphyxia group with no and mild HIE (no/mild-HIE) and the asphyxia group with moderate or severe HIE (mod/sev-HIE) compared to the control group and to the group with other diagnosis (OD).

ALT, AST and LDH predicted HIE with the best predictive value seen for LDH with a sensitivity of 100% and specificity of 97%. The positive predictive value (PV^+) was 87%, the negative predictive value (PV^-) 100% and the likelihood ratio 91.0. A significant difference in

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all three enzymes was seen between the asphyxia groups and the control group but only LDH differed between the group with none/mild HIE and the group with moderate/severe HIE suggesting it is a better prognostic tool.

5 GENERAL DISCUSSION

5.1 Methodological considerations and interpretation of the observations

Paper I

In paper I we showed that asphyxiated newborn infants may develop a transient rise in enzymes similar to that seen in adults. Also, serum ALT and AST values 0-72 hours after birth correlate significantly with moderate to severe HIE, with the strongest correlation at day three. A biochemical parameter that correlates with HIE is of interest since ventilator treatment, sedative drugs and anticonvulsive therapy could make the evaluation of the severity of neonatal encephalopathy difficult.

The three weaknesses of paper I were: (a) that blood samples for enzyme analysis were collected when blood were drawn for clinical routine analysis. As a result, serial enzyme activity was not measured at fixed times during the first 72h. In some cases, the interval between two samples exceeded 24 hours, an interval that could potentially hide a peak value. The long intervals between first and second sample makes it impossible to be sure about the temporal pattern in some of the included cases. More conclusive information about the enzyme pattern in relation to the timing of the asphyctic event might be obtained if the initial sampling is done at birth and the following sample(s) collected within 24 hours, preferably at 6h post partum. (b) There was an absence of samples 0-24 hours after birth in the control group. In healthy newborns, a substantial rise in AST and LDH is seen at 12 and 24h post partum compared to birth (10). These peaks are followed by a decrease at 72h. However, the lowest AST and LDH value during the first week of life under normal conditions is seen directly after birth (10). These substantial alterations in enzyme activity during the first 24h

after birth were missed in our control group making it less representative. Finally, (c) only twenty-six of a total of 48 infants meeting the inclusion criteria were studied. The reasons for non inclusion were forgetfulness and/or high work load in the neonatal ward. Most patients were included by the authors when on duty. However, the eligible patients not included did not differ from the study group in background variables making inclusion bias unlikely. The two different temporal enzyme patterns found in the study is interesting and it is possible that an elevation in serum AST, soon after birth, not followed by any further elevation indicates an antepartum hypoxic-ischemic event. The study was not designed to answer this question but a trend between enzyme pattern and information about the possible onset of the asphyctic event in the maternal record were seen (Table 7) (Fischer's Exact test, $p=0.08$). Still, paper I is, to our knowledge, the first study to explore the individual enzyme patterns seen after asphyxia in newborn infants and thereby contributes with basic knowledge about the AST, ALT and LDH activity during the first week of life and if this activity differs between antepartum and intrapartum asphyxia.

Paper II

The main finding in paper II was that delayed HT was neither neuro- nor organ-protective, nor did either mode of cooling produce more adverse effects than NT. We found no difference between the two modes of cooling in any of the biochemical or histopathological variables investigated.

In studies on fetal sheep (6) and in clinical trials (8, 9), a significant effect was seen with a 5.5h delay in HT treatment. Since the degree of injury was relatively mild initially in our study, we had hypothesized that we would detect a protective effect if present as HT is thought to be more effective in milder injury (8). Previously, Tooley et al (16) found that 24h HT, when started immediately, gave significant neuroprotection in our piglet model. If HT is

applied with a delay, 24h may be insufficient for this species, as is the case in fetal sheep (personal communication, Alistair Gunn). The major weakness of the study was the low median brain pathology score seen in the study. In the normothermic group the median average brain pathology score was 0.43 (IQR, 0.29-2.66) on a 0-4 scale making it difficult to assess the potential neuroprotective effects of HT. A relationship between gender and number of organs with lesions was seen with males suffering fewer damaged organs. This is the first study to show gender differences in MOD after HI in the neonatal period. It is known that organ blood flow is redistributed during HI in animal models (8). One could speculate that the higher incidence of MOD in females reflects a more functional redistribution of organ blood flow and could explain the previously reported gender difference which protects females from neurological damage in the neonatal period (242). In our NT group, the prevalence of MOD was 5/5 in the females and 6/11 in the males. Mean brain pathology score (\pm SD) in the same group was 1.57 (\pm 1.62) in males compared to 0.33 (\pm 0.19) in females ($p=0.078$). This finding is interesting and gender and organ damage should be taken into consideration in future studies of neuroprotection in the neonatal period.

We speculate that the rise in Ca in the cooled groups in our study is due to HT-induced inhibition of cellular Ca influx in affected organs.

Magnesium is known to bind to NMDA channels and thereby block the Ca influx that leads to cell death (243) Magnesium is also important in the first step of the catalytic mechanism of Na^+/K^+ -ATPase (54). Low serum-Mg levels have been seen in cord blood of newborn infants with moderate and severe HIE (244). We speculate that HT treatment in some way mobilizes Mg from the bone structure leading to greater availability of Mg to bind to NMDA channels. The Ca and Mg pattern seen during the cooling period in our study could be a reflection of less compromise of cellular integrity during HT compared to NT. The delay before initiating

cooling and/or the brevity of the HT treatment could explain why no histopathological or biochemical differences were seen across the groups.

Reduced tissue perfusion during HT may be more profound during WBC with a lower temperature in the skin. We did not see any differences in the levels of plasma lactate between the groups. Hence, we have no evidence of diminished peripheral perfusion during either cooling method.

Paper III

The main finding in paper III was that the biochemical markers AST, ALT and LDH did not differ between pigs with histologically verified liver pathology and pigs with normal liver histology.

Global HI is known to cause liver damage in the newborn pig with histological findings similar to the injuries seen in term newborn infants (237-240). Also, in preterm babies, liver necrosis is reported as a post mortem pathological finding (245). The inability for the liver cells to use alanine as a substrate for glucose production is also seen in both newborn term pigs and pre term human babies suggesting a low intracellular ALT activity in both (224) (67). When exploring the activity levels of plasma AST, ALT and LDH after HI, neither newborn pigs nor preterm infants (11, 227) respond to the insult with an enzyme increase. In term infants, on the other hand, the elevation is substantial (9, 11). We speculate that the intracellular enzyme activity in pigs and preterm infants is not mature enough to cause the enzyme elevation seen in term infants, even if the hepatocytes are substantially damaged by HI. A direct analysis of liver tissue activity of these enzymes after HI could solve this question.

In paper III we used animals subjected to HI but without liver histopathology as the control group. The reason for this decision is that both LDH and AST are present in other organ

systems (e.g. muscles, erythrocytes and the heart) (66, 246). Consequently, multi organ dysfunction, which is frequently seen after global HI (2), could contribute to the plasma activity of these enzymes. Therefore, a potentially false relation between liver injury and enzyme activity could be found if a control group had been used that had not been exposed to HI.

An LDH increase at the end of insult was seen in the pathology group, but not in the group without liver pathology, supporting a hepatic origin, even if LDH is present in the whole body. This is an interesting finding showing that LDH responds quickly to HI, even before the onset of reoxygenation/reperfusion and seems to be an immediate and sensitive marker of HI in pigs. A rapid increase in LDH has also been seen in older pigs with a reversible, significant increase as soon as 2min after the end of an episode of warm liver ischemia (247). A significant increase in liver pathology score was seen in the pigs where the UVC tip was found inside the liver. This is an important finding highlighting that iatrogenic origin of liver injures frequently occurs (240).

Paper IV

The main findings of paper IV was that ALT, AST and LDH activity predicted HIE with the best predictive value seen for LDH with a sensitivity of 100% and specificity of 97%. Data were combined from a Swedish unselected cohort of term infants and a combined cohort of infants from UK and Sweden investigated after birth due to the presence of fetal distress or any sign of perinatal asphyxia. Combining infants from different centers is necessary because of the low incidence of HIE in our settings but introduces a necessity to document similarities in the underlying populations and caution in interpretation of the results. However, there are several factors increasing the validity of our findings: The clinical criteria for evaluating the infants (Apgar, HIE) and the clinical laboratory methods (LDH, ALT, AST and pH) are well

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established. Further, the changes in the enzyme levels are large, making minor differences in laboratory standards and the different times for sampling less important. One weakness in our study was that blood for enzyme analysis was collected later in the asphyxiated infants (0.5-12h post partum than in the control group (0 h). The enzyme activity in serum increases by approximately 40% under normal conditions during the first 12h after birth in healthy newborns (226). Therefore, our study may slightly overestimate the enzyme differences between the control and the asphyxia groups. The present results showing that LDH predicts mild, moderate and severe HIE is of direct clinical interest viewed from several perspectives: Firstly, a prognostic tool early after birth would make it possible to select candidates for HT treatment. Secondly, the recent data showing that infants who suffered asphyxia, even if not developing moderate or severe HIE, are at greater risk of behavioural problems at school (de Vries LS, in press) and low intelligence quotients (IQs) latter in life (248). This raises the question whether neuroprotective therapy should be provided for this entire group of infants in the future. A biochemical marker for prediction of mild as well as moderate to severe HIE would be of interest from this perspective.

Thirdly, LDH is a very cheap test and there is a bed side test available needing only one drop of blood for immediate analysis. Based on the fact that the major proportion of the illness caused by asphyxia is seen in developing countries with less resources, a cheap and adequate prognostic tool for assessment of the degree of HIE during the first hours of life is needed. Finally, our results are also of interest in obstetrics where measurement of LDH, ALT and AST in scalp blood during birth could offer a predictive test in fetuses with antepartal asphyxia and fetuses that do not respond to the asphyxia with abnormal pH and lactate (72).

5.2 *Speculations*

Like in the brain, HT treatment significantly prevents or ameliorate hypoxia, ischemia and/or reperfusion injuries in the liver (162, 163, 249). As seen in this thesis, many of the mechanisms leading to cell death in the brain and liver are similar. One thing that the brain and liver also have in common is that the mechanisms for the organ protective properties of HT are largely unknown. A lowered metabolism, reduction in free radical production and inhibition of proinflammatory cytokines have been suggested as protective mechanisms of HT in both organs (160, 198, 250), while attenuation of the release of excitatory amino acids is not discussed in the liver. As far as the author is aware, NMDA receptors are not present in the different types of cells in the liver. So, either the organ protective properties of HT are different between the organs, or the mechanisms of cell death shared by the liver and brain lie in a shared mechanism.

As described previously, there is a limitation of the usefulness of cord blood gas as a predictor of asphyxia as illustrated in the newborn monkey (72). In this model, pH and pCO₂ remained normal during partial asphyxia (where the respiratory gas exchange to the fetus was insufficient for a longer time and diminished gradually). Still, this partial “non-acidotic” asphyxia caused low pO₂ and was followed by white matter injury (72).

These findings do have obstetric implications illustrating the group of fetuses that do not respond with lactate and pH alteration during or after birth, even if severely asphyxiated. In this type of asphyxia, the differing dynamic patterns and decline rates of LDH, ALT and AST could make it possible to identify fetuses entering the delivery with ante partum HI and provide information whether the asphyxia started before or during delivery. A study is now

under way with the primary focus to explore if enzyme analysis can be used for intrapartum surveillance as a complement for the methods in use today.

The studies in this thesis were not designed for investigation of the potential of these enzymes for timing of the asphyctic event. However, the enzyme pattern in cases with known onset of asphyxia in our studies support the theory and resemble those seen in adults after cardiovascular events with known onset (13, 14).

Finally, the author wants to raise the question if the leakages of LDH and aminotransferases have a physiological role during HI. It is known that an efflux of glucose from the liver is seen after HI, glucose that can be metabolised giving ATP (119). Perhaps there is also an increased need for the enzymes catalyzing the metabolic reactions, where the substrates are converted to form ATP. As such, the leakage of intracellular enzymes could have a positive effect on the energy production in extra-hepatic tissues.

6 CONCLUSIONS

- Severe asphyxia can cause hypoxic hepatitis. Furthermore, two different temporal patterns for aminotransferases are seen suggesting an intra respectively antepartum hypoxic event.
- Elevations of AST and ALT during first 72 hours after birth correlates with HIE.
- Twenty-four hours of mild HT using SHC or WBC, induced 3h after a 45min global hypoxic-ischemic insult, have no beneficial effects on organ or brain pathology in newborn pigs.
- Normal plasma activity of AST, ALT and LDH do not exclude substantial liver injury in the newborn pig.
- LDH, ALT and AST are good predictors of the different grades of severity of HIE during the first 12h after birth.

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