Resuscitation of the newborn with 100% O_2 – detrimental effects on the brain, lungs and heart

An experimental study in piglets

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To Ulrik and Hanne

To vers om at ville

Hvis du frygter
for besvær,
kan du liså godt
la vær.

Hvis du uden
vaklen vil,
er det nesten
vundet spil.

Piet Hein

Contents

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Resuscitation of hypoxic piglets with 100% oxygen increases pulmonary metalloproteinases and IL-8. In press in Pediatric Research, 2005.

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Increased myocardial matrix metalloproteinases in hypoxic newborn pigs during resuscitation: effects of oxygen and carbon dioxide. European Journal of Clinical Investigation 2004; 34:459-466

IV **Munkeby,B.H.; Lyng, K., Frøen,J.F., Winther-Larssen,E.W.; Rosland, J.H.; Smith, H.J.; Saugstad, O.D. and Bjørnerud,A.**

Comprehensive morphological and functional MR assessment of early neonatal brain injury in a piglet model.

Journal of Magnetic Resonance imaging, 2004. 20(1):8-15.

Abbreviations

AAPH = 2, 2'-azobis (2-amidinopropane)dihydrochloride

ADC = apparent diffusion coefficient

BAL fluid = bronchoalveolar lavage fluid

β - PE = beta-phycoerythrin

BPD = bronchopulmonary dysplasia

CLD = chronic lung disease

$CMRO_2$ = cerebral metabolic rate of oxygen

DWI = diffusion-weighted imaging

HIE = hypoxic ischemic encephalopathy

HMRS = proton magnetic resonance spectroscopy

LV = left ventricle

MABP = mean arterial blood pressure

MMP = matrix metalloproteinase

MRA = magnetic resonance angiography

MRI = magnetic resonance imaging

OFR = oxygen free radicals

ORAC = oxygen radical absorbance capacity

PaO_2 = arterial O_2 tension

$PaCO_2$ = arterial carbon dioxide tension

pCO_2 = pressure of CO_2

PWI = perfusion weighted imaging

ROS = radical oxygen species

R.F.U. = Relative Fluorogenic Unit

RT-real time PCR = reverse transcriptase polymerase chain reaction

RNA = ribonucleic acid

RV = right ventricle

TIMP = tissue inhibitors of MMP

Background

In 1980, Saugstad and Aasen introduced the concept of hypoxia-reoxygenation injury through oxygen free radicals and suggested that care should be taken when using pure oxygen during resuscitation (1). Since then, researchers at the Department of Pediatric Research, among others, have worked on the intriguing questions concerning oxygen concentration during neonatal resuscitation. Numerous publications and theses have been presented, all adding a little more knowledge to a complex subject. Although oxygen is widely used, more than 200 years after its discovery we still do not know in detail:

1) The optimal oxygen concentration and saturation during resuscitation of the newly born;
2) The short- and long-term effects on morbidity and mortality and on growth and development of using oxygen in the newborn period.

With this thesis, I hope to participate in the ongoing debate on whether to use ambient air, pure oxygen or an intermediate dissolution in neonatal resuscitation.

Introduction

Birth asphyxia

Epidemiology

Approximately 4 million infants out of the 130 million worldwide annual births suffer from birth asphyxia. Of these, approximately 25% die and 25% develop some kind of sequelae (2). Birth asphyxia ranks first among the perinatal insults that cause neurodevelopmental handicap in the newborn, particularly in the full-term baby (3).

Definition

Asphyxia is defined as a condition of impaired gas exchange that leads to three biochemical effects: hypoxemia, hypercapnia and metabolic acidosis (4). Because of the uterine contraction during the normal birth process, all foetuses experience some impairment of gas exchange. The term “asphyxia” should not be used unless the neonate meets all of the following conditions (5):

- Evidence of metabolic acidosis in foetal umbilical cord arterial blood obtained at delivery (pH < 7 and base deficit ≥ 12 mmol/L).
- Apgar scores of 0 - 3 for five minutes or more.

- Evidence of neurological sequelae (e.g. seizures, coma, hypotonia) and one or more of the following organ system injuries: cardiovascular, gastrointestinal, haematological, pulmonary or hepatic injury or renal system dysfunction.

To evaluate neurological sequelae, the combination of magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (HMRS) also makes it possible to evaluate severity and to help predict the outcome of hypoxic-ischemic encephalopathy (HIE) (6).

A diagnosis of the asphyxiating event is associated with the interruption of the oxygen supply or blood flow to the foetus, which can be secondary to problems in the mother (e.g. hypotension, pre-eclampsia, uterine tetany, uterine rupture), the placenta or the umbilical cord (e.g. abruption, infection or inflammation, umbilical cord compression or occlusion), or in the foetus or infant (e.g. central nervous system depression, anomalies, infection) (7).

Hypoxic-ischemic encephalopathy

Birth asphyxia is probably best diagnosed and assessed by what it leads to. The foetus that experiences a significant asphyxial episode is at risk of developing HIE or other organ sequelae. HIE is caused in the full-term and post-term newborn, while in the pre-term newborn unspecific changes occur due to the immaturity of the central nervous system (8). It should be diagnosed only when an infant has clinical findings of an encephalopathy, such as neurological depression or seizures, and has experienced a

severe asphyxiating event (9). The severity of HIE is probably the strongest clinical predictor of outcome in asphyxiated newborn infants. According to the modification of Sarnat and Sarnat (10, 11), HIE is classified in three clinical stages. Mild HIE (grade I) includes irritability, poor sucking and mild hypotonia, and always has a positive outcome. Moderate HIE (grade II) includes lethargy, marked abnormalities of muscle tone, seizures and the need for tube feeding, and leads to severe handicap in approximately 20% of the cases. Severe HIE (grade III) includes coma, prolonged seizures, severe hypotension and respiratory failure, and in most cases results in severe handicap or death (12).

Mechanisms

Hermansen described the acidosis paradox, i.e. a beneficial effect of a mild to moderate acidosis (13). He described three mechanisms that could theoretically limit cerebral damage. Hypercarbia may result in cerebral vasodilatation and increased cerebral flow. Acidosis decreases cerebral metabolism, lowers the cerebral oxidative needs and promotes the unloading of oxygen from the foetal haemoglobin by shifting the oxygen dissociative curve. These protective mechanisms can be lost with severe acidosis, which further decreases cardiac output and secondary cerebral ischemia (14). When the pH decreases below 7, the risk of developing long-term cerebral damage increases (15). When asphyxia occurs, the organism sets off the necessary mechanisms to preserve vital organs (brain, heart, and adrenal glands), while other organs such as the kidney, lungs, gastrointestinal tract and skin are affected to a greater or lesser degree depending on the duration of the episode (16). When compensatory mechanisms fail, the brain is affected

by HIE. The cardiovascular system is affected by myocardial ischemia or cardiac stunning, poor contractility due to acidosis, tricuspid insufficiency (often from pulmonary hypertension) and hypotension (17).

Brain injury secondary to hypoxic-ischemic disease is the predominant form encountered in the term infant (18). Cell damage may occur both during the ischemic phase and upon reperfusion, probably by different mechanisms (19). The normal function of the brain inevitably depends on an adequate supply of oxygen and glucose. Acute reduction in cerebral oxygen delivery leads to the breakdown of neuronal energy metabolism within few minutes (20). When the cerebral blood flow is compromised, brain cells are deprived of oxygen and glucose, resulting in a decrease in high energy phosphate (ATP) levels. Ionic gradients across the cell membrane cannot be maintained, partly due to loss of Na^+/K^+-ATPase activity. Influx of Na^+ and Ca^{2+} into the cell is accompanied by water, resulting in cytotoxic oedema. These changes may become irreversible and lead to cell necrosis and brain infarction as a result of ischemia alone. Reperfusion may temporarily correct the energy failure, but it may trigger a chain of events leading to delayed neuronal death or secondary damage due to brain swelling (19). The acute lack of cellular energy during ischemia induces almost complete inhibition of cerebral protein biosynthesis.

A second wave of neuronal cell damage occurs during the reperfusion phase induced by the post ischemic release of oxygen radicals, the synthesis of nitric oxide (NO), inflammatory reactions and an imbalance between the excitatory and inhibitory neurotransmitter systems (21). Knowledge about these pathophysiological mechanisms has enabled scientists to develop new therapeutic strategies that have been shown to be neuroprotective in animal experiments. Promising therapeutic effects are seen by the post

ischemic induction of mild cerebral hypothermia (22, 23), the application of the calcium-antagonist flunarizine, and the administration of magnesium (24-26).

Chronic lung disease

Definition and epidemiology

Bronchopulmonary dysplasia (BPD), now more commonly referred to as chronic lung disease of infancy (CLD), was first described by Northway and colleagues in 1967. CLD is defined as chronic oxygen dependency in newborns at 36 weeks post-menstrual age (27). The incidence of CLD in infants with a birth weight between 500 and 1500 g ranges between 3% and 43% (28). CLD affects almost half of all ventilated infants below 32 weeks' gestation, and contributes significantly to the long-term morbidity of preterm infants (29). Despite improvements in neonatal intensive care over the last ten years, including the widespread use of exogenous surfactant and antenatal steroids, there has been little alteration in the incidence of this disease (30). This is believed in part to be due to the improving survival of extremely preterm infants who now proceed to develop CLD. It has also been noted that the nature of CLD has altered. Previously, CLD affected larger infants with severe Respiratory Distress Syndrome (RDS) who were exposed to high ventilatory pressures and oxygen concentrations, and were characterised by an inflammatory response to this treatment. Now CLD affects extremely preterm infants who may not have significant RDS at birth but have immature pulmonary development.

Exposure to the extra-uterine environment and to treatment modalities result in abnormal lung development (31).

Mechanisms

The development of CLD has been associated with the use of hyperoxic treatment during ventilation. Inflammation has been shown to contribute to the development of this disease both on histological examination of diseased lungs and by the use of bronchoalveolar lavage (32). Hyperoxia is believed to contribute to this inflammatory process by causing direct injury to epithelial and endothelial cells. The formation of reactive oxygen species is thought to result in a cytokine production and vice versa (33). They act within a complex network and orchestrate an inflammatory response. The mechanism, by which this disease process occurs, has become progressively elucidated with time by the use of in vitro and *in vivo* investigations. Preterm infants are particularly vulnerable to oxygen toxicity as a consequence of an immature antioxidant system. Complex cytokine networks play a crucial role in the pathophysiology of CLD and provide a target for therapeutic modulation. Pulmonary metalloproteinases may also be activated in the early stages of CLD (34, 35).

Treatments

A number of treatments have been investigated which down-regulate the immune response in a non-specific manner e.g. corticosteroids and non-steroidal anti-inflammatory drugs. However, these treatments have many undesired side-effects that limit their usefulness (30).

Cardiovascular dysfunction in birth asphyxia

Cardiovascular dysfunction related to birth asphyxia was first described in the 1970s (36). The incidence is not well known, but cardiac abnormalities should be suspected in seriously asphyxiated infants. In most cases these are reversible within a few days without any treatment. Severe hypoxic myocardial dysfunction most often occurs in seriously asphyxiated children, usually in combination with other serious organ failures that mask the cardiac problem. When an asphyxic event occurs, the organism sets off a series of mechanisms to ensure that vital organs such as the brain and heart continue working normally. When the event prevails in spite of compensatory mechanisms, it may affect the vital organs.

Myocardial dysfunction, hypotension and increased pulmonary vascular resistance are well-known consequences of hypoxic-ischemic insults in neonates and are related to low alveolar oxygen tension and increased pulmonary vascular resistance (37). The abnormalities can be classified as transient tricuspid regurgitation (TR), transient

myocardial ischemia (TMI), transient mitral regurgitation (MR), and persistent pulmonary hypertension of the newborn (PPHN) (17).

1) TR has been described as the most common cause of cardiac murmur in the newborn during the first days of life. It is most often associated with PPHN (persistent pulmonary hypertension of the newborn), both probably being due to asphyxial damage. It tends to disappear after a few days and seldom causes long-term problems. Isolated TR needs no therapy unless associated with TMI or PPHN.

2) In the most severe cases TMI may cause an acute myocardial infarction (8). TMI should be suspected in an asphyxiated newborn with respiratory distress and poor pulses - especially if a murmur is detected. Cardiac Troponin I have been shown to be a sensitive and specific marker of myocardial damage in both the adult and pediatric population (38, 39)

3) MR is far less common than TR and is often part of transient myocardial ischemia. This condition is recognised as a pan systolic murmur of the apex, and may reduce the left ventricular function. MR requires therapy more often than TR as it is commonly associated with TMI and left ventricular dysfunction.

4) Persistent pulmonary hypertension of the newborn (PPHN) is a condition in which the hemodynamics of fetal life are maintained after birth. The pulmonary vascular bed remains constricted and there is right to left shunting across the ductus arteriosus and foramen ovale. Perinatal hypoxia is one cause of the development of PPNH.

Apgar score

The Apgar score - a widely used method for the immediate evaluation of the newborn infant's condition - was first developed in 1952 by Dr. Virginia Apgar, an obstetric anaesthesiologist (40). Using signs traditionally observed by anaesthesiologists, her goal was to develop a scoring system that could assess a neonate's transition after birth. It was developed as an objective tool which measures five signs of physiologic adaptation. The score is based on the sum of the values assigned to the infant at one and five minutes of life, with a score of seven or more indicating that the baby is in good to excellent condition (9). The initial intent of the Apgar score was to predict survival and not perinatal asphyxia. Although the Apgar score can to some extent predict mortality, it is not a tool to be used alone in determining the neurological outcome of infants who survive. Low Apgar scores estimated during the first few minutes of life are not specific to any particular condition and are poor predictors of brain injury (41-43). The score does not take into account preterm infants or intubated infants who often gain lower scores based on these situations. It has also been suggested that it is antiquated due to the modern application of resuscitation and neonatal care (9).

Normal blood oxygen saturation during the first few minutes of life is below 85%. A one minute Apgar score of nine or ten is probably not ideal and should not be a goal. Indeed, the Apgar score might have contributed to the subsequent excessive use of oxygen in newborn infants (44).

Oxygen in neonatal medicine

Karl Scheele and Joseph Priestly discovered oxygen independently in 1772 and 1774. Priestly realised that this gas was not only life-giving but might be poisonous as well "The new air might not be proper for use in the healthy states of the body…the air which nature has provided us may be as good as we deserve". Shortly after its discovery, oxygen was used for medical purposes. In 1780, Chaussier in France administered oxygen in order to establish normal breathing in newborn infants and in the years to come oxygen as a therapeutic agent was tested out in a number of conditions.

The gas was administered by various techniques (subcutaneous injections, iv, intragastrically) in order to resuscitate premature infants and to manage attacks of apnoea. In 1893 Thomas Morgan Rotch proposed that oxygen should be applied two or three times each day into the incubator of newborn infants as a "stimulant". In 1928 Flagg described a procedure for intubation and intermittent positive pressure insufflations using a mixture of oxygen and carbon dioxide for resuscitating asphyxiated newborns (45). Routine oxygen therapy for newborn infants was introduced in the United States in the 1930s in order to improve the respiratory pattern and to reduce a supposed risk of brain damage caused by unrecognised lack of oxygen (46). After 1945 incubators were used to maintain a high oxygen concentration. From a biochemical point of view it seemed logical and intuitive to reverse the anaerobic state as quickly as possible with 100% oxygen since 18-19 times more ATP is produced from glucose during aerobic metabolism than during anaerobic metabolism. However, following the discovery of the link between retinopathy of prematurity, chronic lung disease (CLD) and the liberal use

of supplemental oxygen therapy, controversy has surrounded the use of oxygen in neonatology (47, 48).

Is it harmful to resuscitate neonates with pure oxygen?

In the past 12 years, neonatal research has concluded that if assisted ventilation is required, room air is as efficient as 100% oxygen for newborn resuscitation (49, 50). Clinical and experimental studies have also concluded that resuscitation with oxygen might have detrimental effects (51, 52). A meta-analysis published in the Lancet (53) even suggested that mortality is lower in newborn infants resuscitated with room air compared with those resuscitated with 100% oxygen, with one death being prevented for every 20 babies resuscitated with air versus 100% oxygen. The recent meta-analyses (53, 54) investigating data over 12 years of translational research strongly suggests that our practice of resuscitating term infants who need positive pressure ventilation in 100% oxygen should in most cases be avoided. Neonatal medicine and newborn resuscitation guidelines and practice need to be revised globally.

Neonatal resuscitation

Rapid and complex physiological changes occur during birth. The neonate has to make the transition from a fluid-filled environment in which the placenta serves as the gas-exchange organ for the foetus to an air-filled environment in which the neonate's own cardiopulmonary system has to function independently within minutes of birth for survival. Amazingly at least 90% of neonates successfully make this transition without

the need of help. The remaining 10% of newborns require some assistance to begin breathing at birth, and about 1% may require intensive resuscitation efforts (55, 56). Thus resuscitation of the newly born infant is one of the most widely practiced medical procedures, and oxygen is the most commonly used remedy in neonatal units as an integral part of respiratory support (57). Of course, the aim of resuscitation is to prevent neonatal death and adverse long-term sequelae.

The optimal concentration of oxygen for neonatal resuscitation is uncertain. Guidelines from the American Heart Association and the American Academy of Pediatrics (2000) recommend that pure oxygen should be used during initial newborn resuscitation whenever positive pressure ventilation is required (58). This practice has been challenged by other experts in the field, on the basis that insufficient scientific evidence exists to support it. The neonatal resuscitation program currently recommends discontinued resuscitation if heart rate is absent after 15 minutes of appropriate resuscitation. This is based on data that newborns who are asystolic at ten minutes of life usually die, and if they survive they have severe disabilities (59-62). New guidelines for the resuscitation of depressed newborn infants, which were discussed at the conference "2005 International Consensus on cardiac pulmonary resuscitation (CPR) and emergency cardiovascular care (ECC) Science with Treatment Recommendations Conference" in Dallas, January 2005, will be published later this year. Defining the optimal oxygen concentration and technique for neonatal resuscitation is an extremely important challenge and has the potential to improve neonatal outcome globally.

Perinatal hypoxic-ischemic tissue damage

Oxidative stress

Birth is accompanied by an increasing oxidative stress as birth itself is a hyperoxic challenge. The foetus is moved from an intrauterine hypoxic environment with a pO_2 of 2.6-3.3 mmHg to an extrauterine environment with a pO_2 of 13.3 mmHg. The link between oxygen and the generation of oxygen free radicals (OFRs) was described 50 years ago (63).

OFRs are highly cytotoxic molecules generated during the restoration of oxygenated blood flow following ischemia or hypoxia (64). They are cytotoxic because they have the ability to interact with and alter the principal components of cells including proteins, lipids, carbohydrates and DNA (65-68). They also play important roles in normal biological processes, apoptosis and necrosis (69).

In 1988, Saugstad introduced the term "oxygen radical disease in neonatology", suggesting an important role of OFR in the generation of different neonatal morbidities (70). Animal and human studies have shown an increasing OFR production with higher levels of inspired oxygen and degree of prematurity (71, 72). Perinatal asphyxia is a hypoxic-ischemic event, and with subsequent resuscitation infants are at risk of OFR-related injury to the vital organs (73). They are implicated in the pathogenesis of many neonatal diseases such as perinatal asphyxia, chronic lung disease (CLD), retinopathy of prematurity (ROP), necrotizing enterocolitis, interventricular hemorrhage, periventricular

leucomalacia, pulmonary hypertension, persistence of ductus arteriosus and myocardial dysfunction (64, 74-79).

The lungs are directly exposed to the highest partial pressure of inspired O_2 and, together with its large surface area and blood supply, they are susceptible to injury mediated by OFR. Pulmonary damage as a result of oxygen exposure is an important clinical complication in patients treated with high levels of oxygen (67). The initial phase of pulmonary oxygen toxicity is characterised by damage to airway and alveolar epithelium and capillary endothelium. This leads to interstitial and alveolar oedema followed by marked neutrophil infiltration into the tissue. The second phase of oxygen toxicity is characterised by proliferation of alveolar type II cells and after prolonged exposure by interstitial fibrosis (80). This results in permanently impaired gas exchange. Oxygen-induced lung injury is manifested as CLD in premature infants. The second category of toxic effects induced by oxygen is indirect. The neonatal brain, in particular the preterm brain, is vulnerable to oxidative damage due to its high concentration of unsaturated fatty acids, its low concentration of antioxidants and the availability of redox-active iron (81). However, as OFRs in abundance can be harmful, they also play important physical roles, which include signal transduction and the regulation of cellular growth and differentiation (82) and vasoactive control (83), and they are involved in the defence against infection and in mediating immune responses (84-86).

Matrix metalloproteinases

It has previously been reported that oxygen free radicals (OFRs) induce gene expression of several matrix metalloproteinases (MMPs) (87). Several studies have documented

early up-regulation of MMPs in acute disease processes, such as ischemia-reperfusion and hyperoxia in the brain, lungs (35) and heart (88-94). MMPs are a group of zink-dependent endopeptidases involved in the process of tissue remodelling through the degradation of the extracellular matrix (ECM) (95). Tissue remodelling occurs in various physiologic conditions such as embryogenesis and wound healing, as well as in pathologic conditions, including inflammatory diseases, tumor cell invasion and angiogenesis (96). The activity of MMPs is regulated by several types of inhibitors, the most important of which are the tissue inhibitors of metalloproteinases (TIMPs) (97). The role of oxidative stress and its toxic effects on lipids, as well as on the disruption of extra cellular matrix through the up- regulation of MMPs is well established (98-100). Uncontrolled expression of MMPs can result in tissue injury and inflammation (95, 101). In myocardial tissue, MMP-2, activated by oxidants, can cause a detrimental effect on the myocardial contractile function after reperfusion caused by its action on the troponin and contractile mechanism (92, 102-104).

Glycerol

Degradation of membrane phospholipids is a well-known phenomenon that is thought to underlie the disturbance of vital cellular membrane functions in acute brain injuries. Glycerol is an integral part of the hydrophilic portion of the bilayer of glycerophosphate and fatty acids that constitute most cell membranes. Hypoxia-ischemia (HI) increases intracellular calcium, which will activate phospholipases and thereby degrade the bilayer into fatty acids, phosphate and glycerol. Interstitial glycerol is a sensitive and reliable

marker of cell damage in experimental cerebral ischemia (105-107). In the hypoxic term piglet model, the corpus striatum becomes selectively vulnerable (108).

Interleukin-8

In pulmonary tissue, IL-8 is synthetised by circulating monocytes, alveolar macrophages, T-lymphocytes, type II pneumocytes, epithelial and endothelial cells (32). Hyperoxia is known to stimulate the alveolar macrophages to release chemokines such as IL-8 into the alveolar space (90) thereby up-regulating MMP-2 and MMP-9 production (109). Overproduction of pro-inflammatory cytokines has been suggested to be a major factor associated with pulmonary damage. *In vivo*, IL-8 is abundantly expressed in the lungs of animal models during oxygen injury (110) and in human premature infants developing CLD (111, 112). Earlier studies have noted a significant rise in IL-8 levels in broncho alveolar lavage (BAL) fluid (113, 114). Munshi (115) noted a significant rise of IL-8 in tracheal aspirates one to three days postnatally in babies with RDS progressing to CLD. In clinical studies, increased levels of pro-inflammatory cytokines (i.e. IL-8) in BAL fluid, correlated with the degree of pulmonary dysfunction and predicted the development of chronic lung disease in premature infants (116).

Magnetic resonance imaging

Magnetic resonance imaging (MRI) is an attractive diagnostic modality in the developing brain because of the high contrast resolution of this technique combined with its relative non-invasiveness (117). MR perfusion and diffusion weighted imaging (PWI and DWI)

have proved to be sensitive tools for the early detection of brain injury (118-121). The almost instant reduction in microscopic tissue water diffusion following cerebral ischemia, demonstrated with DWI in cats as early as 1990, is well documented (122). The mechanism behind the diffusion reduction during ischemia is still not fully understood, but it is most commonly thought to be caused by cytotoxic oedema which results from the breakdown of the cellular membrane Na^+/K^+-ATPase pump system (123). DWI has lately been shown to be a very sensitive method for diagnosing severe neonatal HIE (124, 125). The use of cerebral PWI is a relatively new diagnostic method, but is already well established in the diagnosis of brain ischemia. In particular, the combination of PWI and DWI has been shown to be a very sensitive and non-invasive method for identifying the ischemic penumbra as the area of mismatch between perfusion and diffusion changes (126). PWI (in terms of relative perfusion maps, rCBF) directly reflects the area of perfusion reduction, and therefore the area at risk of infarction, whereas the region with decreased water diffusion is thought to reflect the area that is irreversibly damaged. The availability of a well-controlled piglet model is of great importance for many types of studies aimed at investigating neonatal injury due to HI. Such a model requires a sensitive method in order to assess the degree of functional changes caused by the induction of HI. Another important aspect is to have a sensitive imaging method to validate that total carotid occlusion has indeed been achieved. By contrast, this could be performed enhanced MR angiography (MRA), possibly combined with immediate perfusion imaging.

Aims of the study

1. To explore whether resuscitation with 100% oxygen compared with ambient air increases acute cerebral, pulmonary and myocardial damage.

2. To explore whether alterations in the $PaCO_2$ - level during resuscitation influences the cerebral, pulmonary and myocardial tissue.

3. To assess if morphological and haemodynamic magnetic resonance imaging in the brain are sensitive tools for the detection of early neonatal brain injury.

Methodological considerations

Animal model

All the experimental protocols were approved by the Rikshospitalet University Hospital's ethics committee for animal studies under the surveillance of the Norwegian Animal Research Authority, and performed by certified FELASA (Federation of European Laboratory Animal Science Associations) category C researchers. All efforts were made to:

- Reduce the number of included animals as much as possible while retaining the scientific requirements for statistical analysis.
- Refine the models so as to minimise the group sizes while maximising the quality of acquired information.
- Replace *in vivo* analysis by in vitro models when this would give equivalent information. This has not been possible in this thesis.
- Relieve animals from any distress by careful handling and adequate analgesia and anaesthesia when needed.

We have used 12-36 h old Noroc (LYxLD) pigs. These are a crossbreed between Norwegian Landrace (L) ½, Yorkshire (Y) ¼ and Duroc (D) ¼. They were transported to the laboratory by the farmer on the day of the experiment. Exclusion criteria were reduced general condition, wounds, dehydration, Hb < 5 g/dL, and weight less than 1200 g. Anaemia in the newborn piglet is a familiar problem. Attempts to prevent the

development of anaemia in piglets by treating pregnant sows with iron show no effect (127). Total blood volume in a piglet is 70 ml/kg body weight and it is possible to withdraw 10% blood on the day of the experiment, excluding piglets less than 1200 g. To reduce the number of piglets we had to exclude, it was important to collaborate with the laboratory animal breeder. He had to be able to recognise the exclusion criteria and take them into account during breeding. The breeder's routines for animal selection were improved.

- Piglets with any of the above-mentioned visible exclusion criteria were not selected by the breeder.
- The piglet was taken from the sow as late as possible on the day of the experiment to avoid dehydration and stress. Piglets become easily become stressed by rapid changes in environments such as being withdrawn from the sow.
- The piglets were transported in a warm incubator to avoid hypothermia.

It is difficult to study the pathophysiology of neonatal asphyxia in humans. Much of our current understanding concerning neonatal asphyxia derives from studies conducted in animal models (128, 129). An important prerequisite for the evaluation of an animal study is to know the possibilities and limitations of the experimental model. The best animal model of neonatal hypoxic ischemic cerebral, pulmonary and cardiac injury is presumably the one that most closely approximates the injury found in human infants. Over the last few decades, a large body of data has been gathered on newborn pigs. The size and body weight is makes this an attractive and workable model. The same type of

equipment usually used in the neonatal intensive care unit may be used during experiments. Their anatomy and physiology are in many areas close to that of humans (130).

However, both in human infants and in animal models, the localisation and extent of the injury observed will not only depend on the degree and duration of the insult, but also on the maturity of the brain, lungs and heart (131, 132). Animal models will always be an approximation to the clinical situation. Thus, the present work cannot be directly related to the resuscitation of asphyxic newborn infants.

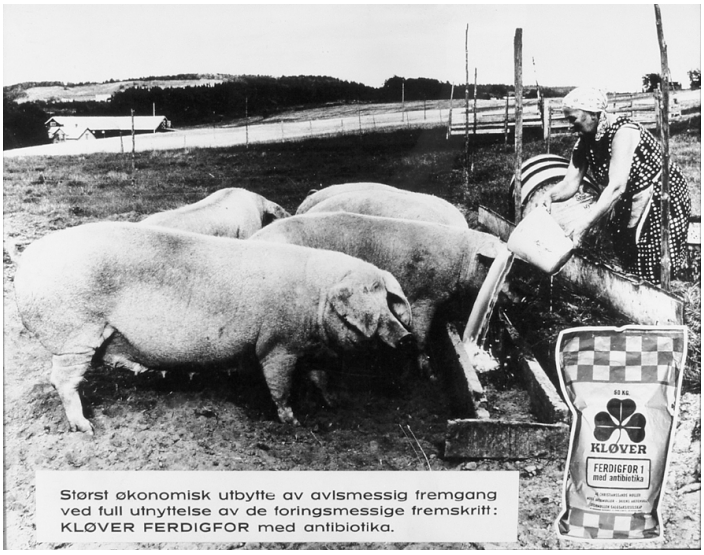
It is important to take into considerations the differences between the species in terms of O_2 responses, different biochemical responses, lack of reference values for common functional variables and different maturation at birth. We used a piglet model since its anatomy and physiology are quite similar to those of humans (130, 133). This model is time-consuming and it is expensive to obtain a large enough series for satisfactory statistical power. It must be emphasised that the animals in the present work were 12-36 h old and were therefore to some extend adapted to extra-uterine life. Thus, whether our findings can be applied to the resuscitation of asphyxiated newborn infants should be determined through clinical trials.

The piglet model is commonly used in medical research due to the similarities of the brain to the human brain (134). Studies have shown that the grey/white matter distribution, changes in brain morphology during development, and sequence of nervous system maturational changes, are comparable for pigs and humans (135). Also, the overall shape and gyral pattern of the piglet brain is similar to that of humans (136, 137). The histological maturation of the brain of a newborn piglet is considered to be

approximately the same as that of human infants of 36-38 weeks of gestation (138), but its physiology is probably more mature. Newborn piglets also have higher rates of cerebral metabolism and cerebral blood flow (CBF) than humans. Normal CBF has been found to be the highest in neonatal pigs and decreasing with age (139), which is in contrast to humans.

Haworth et al studied the adaptation of pulmonary circulation to extra-uterine life in pigs and concluded that functionally the pulmonary circulation did not appear to be mature until the age of two weeks and that an adult pattern was established by the age of six months. The functional changes occurring in pulmonary circulation during the first two weeks of life follow a similar time course to those in the human infant (140).

The cardiovascular system is rather similar to that of humans (133, 141). The ductus arteriosus is functionally closed within 4-20 h after birth.



My grandmother feeding the pigs in 1953.

Anaesthesia

The use of conscious animals in our studies would be unethical and of course not possible since the piglets easily become stressed during surgery. The use of anaesthetics is an absolute necessity, but represents a problem as the drugs may act as confounders. They may complicate the interpretation of results and make comparison with previous experiments difficult. However, the well-being of the animal must always be the first priority.

Anaesthesia was induced by Halothane 4% (Fluthane ® ZENECA). An ear vein was cannulated, halothane was disconnected, and the piglets were given Pentobarbital sodium 20 mg/kg and Fentanyl 50μg/kg iv as a bolus injection. In papers I-III anaesthesia was maintained by a continuous infusion of Fentanyl (50μg/kg/h) and Midazolam (0.25mg/kg/h) (IVAC P2000 infusion pump). In work IV, infusion pumps had to be avoided because of artefacts on the MRI images. The anaesthesia was therefore maintained during the experiment with a continuous inhalation of isoflurane (Abbot Scandinavia AB, Kista, Sweden) (1-1.5% MAC), and a mixture of N_2O (30%) and O_2 (70%) and an hourly bolus injection of Fentanyl 50 μg/kg. A continuous iv infusion (Saline 0.7% and Glucose 1.25%, 20ml/kg/h) was given throughout each experiment. The depth of anaesthesia was monitored by response to painful stimuli elicited by pinching the nasal septum in addition to monitoring heart rate and blood pressure. If necessary, a bolus of Fentanyl (10μg) or Midazolam (1mg) was added. Before and during the experiment, we provided normal rectal temperature in the piglet between 38.5 ºC and 39.5 ºC using a heating blanket and lamp.

Halothane decreases cerebrovascular resistance and increases cerebral blood flow (CBF) in a concentration-related manner provided auto regulatory limits are not exceeded. As a result, intracranial pressure (ICP) may increase. Cerebral auto regulation is lost when the concentration of Halothane increases (142). Halothane was only used for a few minutes to minimise influence on the results.

In addition, the opioid Fentanyl was used as analgesia which in newborn piglets increases cerebral fractional oxygen extraction (143). Pigs require larger concentrations of morphine than dogs or other types of primates (144). Fentanyl dosages acceptable for humans may be inadequate for pigs (145). Studies have shown that, between the species, there are differences in the pharmacodynamic properties of opioid agonists (144, 146). One study concluded that Fentanyl infusions of 50-200 μg/kg/h reduced the minimum alveolar concentration of isoflurane in pigs (147). Fentanyl dosages used in research should therefore be evaluated carefully by the investigators to ensure minimal distress to the animal and to prevent the collection of misleading data (148).

Pentobarbital sodium is an intermediate-acting barbiturate and is probably the most used anaesthetic drug in animal research. Barbiturates have been used as sole agents for anaesthesia in pigs. In the presence of pain, barbiturates can even induce hyperalgesia (149, 150). Unstable haemodynamic conditions will be produced by the large doses necessary to obtain sufficient analgesia (151-153). Pentobarbital sodium should be combined with an analgesic agent during painful procedures. Barbiturates have a dose-dependent cardiovascular depressant effect which is well tolerated in pigs (142, 150). Barbiturates reduce CBF, intracranial pressure, cerebral metabolic rate of oxygen ($CMRO_2$) (154, 155) and have anticonvulsant effects (149). Barbiturates also have a

neuroprotective effect on ischemia-induced brain damage (156, 157). Small bolus dose levels were used at the beginning of the anaesthesia and at the end to kill the piglet. The dose level used is not expected to influence the results.

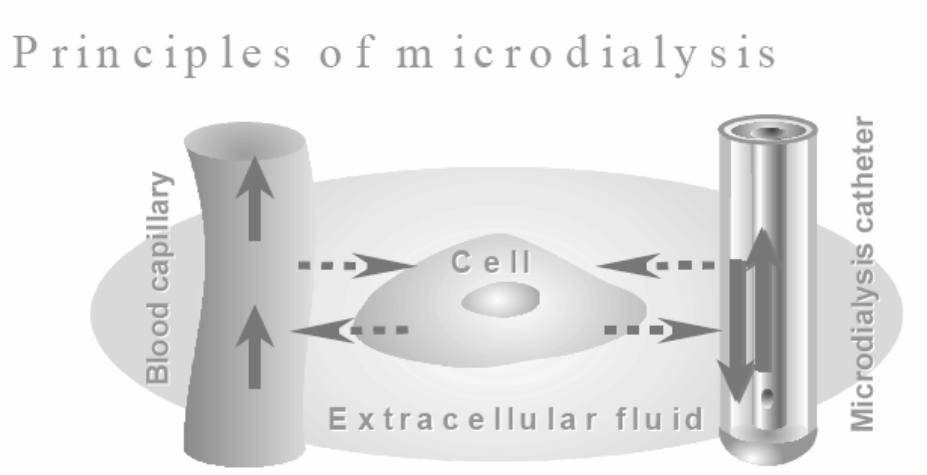
Midazolam is an effective sedative in pigs and has minimal effects on the cardiovascular system (158). Continuous infusion of Midazolam may, however, compromise the cerebral perfusion and oxygenation in piglets (159).

In paper IV the combination of isoflurane/N_2O inhalation and bolus injections with Fentanyl iv were used. The inhalation agent isoflurane as a sole agent or in combination with nitrous oxide (N_2O) has been demonstrated to provide the least cardio depressant effects while providing stable anaesthesia. Pigs are relatively resistant to the analgesic effects of N_2O, which should therefore not be used as the sole analgesic agent during surgical procedures (133). The anaesthetics that were used could influence the metabolism and blood flow in brain, lungs and the heart, but this could not explain the differences between the groups.

Dose levels of anaesthetics for research animals have been developed through veterinary practice and from dose levels used in humans without considering variations between species (153, 160). Earlier, veterinary practice used lower doses if the animal was used for human consumption. Since required dose levels may differ considerably, care should be taken when applying anaesthetic protocols developed for humans to other species, such as pigs.

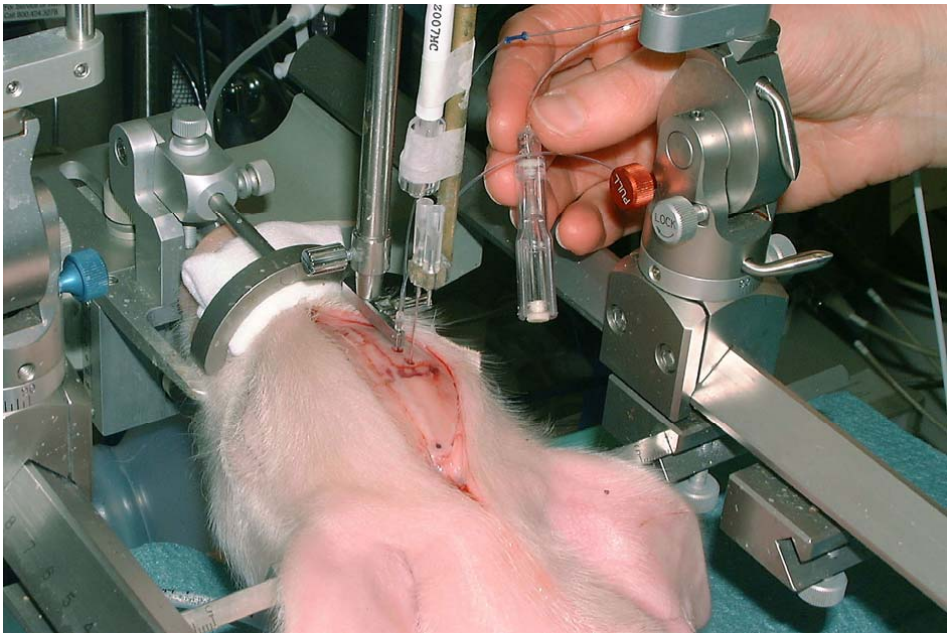
Microdialysis

In vivo microdialysis is a technique used to sample extracellular substances from intact tissue. Microdialysis was invented in the 1970s, and the microdialysis probe was originally developed as an attempt to mimic the passive function of a capillary blood vessel by perfusing a thin dialysis tube implanted into the tissue. The technique can be performed in almost any organ and tissue (161). Initially used in animal studies, the technique is now being used on humans, especially in neurosurgical patients (162-165). The microdialysis probe is continuously flushed by artificial extracellular fluid, and consists of a thin membrane perforated with holes of a specific diameter ("cut-off value" for molecules that can, or cannot pass through). Inside the probe, a thin needle extracts the dialysate, which can be analysed as illustrated below.



Usually, microdialysis measures only a fraction of the actual concentration of the substance in question. As the probe is constantly perfused, the dialysate is removed and a

concentration gradient is created from the extracellular fluid to the dialysate. The concentration of the collected substance is therefore always lower in the dialysate than in the extracellular fluid. The dialysate/extracellular concentration ratio expressed as a percentage is called the microdialysis extraction fraction or *in vivo* recovery. The diameter of the holes in the dialysis membrane is in general so small that it excludes larger molecules, such as enzymes, that would break down smaller molecules that are essential to metabolism or signalling. Thus, the dialysate from the probe is thought to accurately reflect the extracellular levels of these small molecules. However, as dialysis is based on passive diffusion, the flow rate at which the artificial extracellular fluid passes the membrane is crucial in determining the ratio between the true extracellular levels and the levels in microdialysate samples. The absolute recovery (mol/time unit) of substance from the tissue depends on the "cut off" of the dialysis membrane (usually defined as the molecular weight in Daltons, at which 80% of the molecules are prevented from passing through the membrane), the length of the membrane, the flow of the perfusion fluid and the diffusion coefficient of the compound through the extracellular fluid. A flow rate of 1μL/minute was used in these studies. Tissue temperature, blood flow and the metabolism of the collected substance may also influence an *in vivo* recovery. The introduction of a probe into the tissue will always cause damage and the recovery of function will take a certain period of time. An hour is often used to reach baseline conditions. Microdialysis probes were mounted on the stereotactic frame and inserted into corpus striatum as seen below. The analysis of microdialysate has been performed in a CMA 600 Microdialysis Analyzer. The illustration is reprinted with permission from CMA/Microdialysis AB.



Microdialysis as described above.

Biological markers and imaging techniques

Matrix metalloproteinases

The expression of MMPs can be analysed by several techniques. Total MMP activity is an analysis of the joint capacity of MMP-1, MMP-2, MMP-7, MMP-8, MMP-9, MMP-12 and MMP-13 using a fluorogenic peptide substrate (cat no: ES001, R&D systems). The total activity of these enzymes is expressed by Relative Fluorogenic Unit (R.F.U.). A widely used technique is substrate zymography, which identifies MMPs by the

degradation of their preferential substrate and by their molecular weight. Using this technique, it can be determined whether the MMP is in active or latent form. To localise MMPs in tissue sections *in situ* zymography can be performed.

Gelatin zymography

Gelatin zymography is based on the following principles: 1) during electrophoresis, gelatine is retained in the gel, 2) MMP activity is reversible inhibited by SDS during electrophoresis, and 3) the SDS causes the separation of MMP-TIMP (tissue inhibitors of metalloproteinases) complexes during electrophoresis. Another advantage of gelatine zymography is that both the proenzyme and the active forms of MMPs can be distinguished on the basis of their molecular weight (166). Because gelatine zymography only indicates whether the proenzyme displays in the presence of TIMP *in vivo*, it is uncertain how much activity the active form would display in the presence of TIMP *in vivo* (166). Furthermore, the digestion of gelatin by pro-MMPs is somewhat reduced because the latent form still retains its propeptide domain.

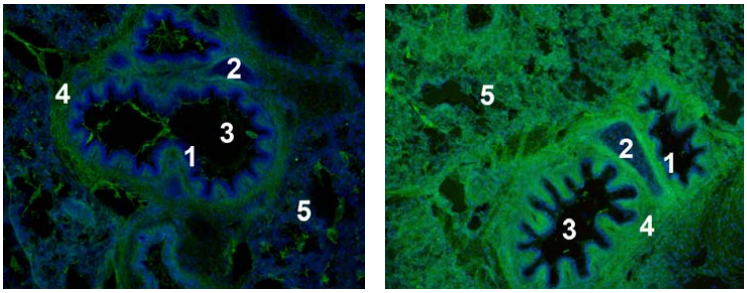
Gelatin zymography is mainly used for the detection of the gelatinases MMP-2 and MMP-9. It is extremely sensitive so that levels of 10 pg of MMP-2 can be detected (167). It is an adaptation of substrate zymography that is suitable for frozen sections. The nature of the substrate dictates which MMP can be detected (168). In addition, the extraction procedures can artifactually activate enzymes or result in the interaction of active enzymes with their respective inhibitors, which may have been localised in distinct compartments in the intact cells or tissue. Likewise, if a specific protease activity is localised in a relatively small part of a diseased tissue, it may not be detected because of

its dilution in the entire tissue extract. For these reasons, techniques to localize specific proteolytic activity in tissue sections may provide crucial additional information on the exact role played by certain proteases in various physiologic and pathologic conditions. To identify the gelatinases in our gels we used a human and molecular weight standard. At that time there was no pig standard available to identify the zymography-bands, which would have made our identification more precise.

In situ zymography

In situ zymography (ISZ) is a relatively low-cost technique that uses a substrate which deposited on or under a frozen section of an unfixed tissue sample. During incubation, the substrate will be digested by the activated MMPs in a time- and dose-dependent manner and offers the ability to estimate different protease activities in combination with the localisation of these activities in tissue sections (169). Only active MMPs are detected. The degradation of the substrate is detected by light microscopy or fluorescence microscopy, depending on the type of substrate (170). A limitation of this technique is the difficulty to discriminate between the different classes of MMPs. The MMPs are detected using a photographic emulsion or a fluorescent substrate. ISZ is a useful tool which can be used to localise protease activity in tissue sections in an inexpensive and rapid manner. A disadvantage of the photographic method is the difficulty to standardise the method (171).

***In situ* zymography showing net gelatinolytic activity in pulmonary tissue.**



1) Epithelium
2) Cartilage
3) Bronchial lumen
4) Submucosa
5) Alveoli

Baseline **Resuscitation with 100% oxygen**

Analysis of gene expression

Total RNA was isolated using silica-gel based membranes (RNeasy columns). All RNA samples were treated with DNase to eliminate the contamination of small amounts of DNA. The concentration and purity was evaluated by measuring the absorbency at 260/280 nm and the integrity of total RNA by the use of agarose gel electrophoresis. Only samples with high integrity and purity were used in the experiments.

To identify and quantify gene expression of specific transcripts we used real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR). This is a sensitive method for the detection of low-abundance mRNA. In contrast to conventional RT-PCR, the accumulation of the PCR-product is monitored for each cycle using a fluorogenic sequence specific probe (e.g. TaqMan probes). This makes it possible to collect the data in the exponential phase of the amplification, obtaining reliable quantitative results dependent on optimising internal standards.

To correct for sample-to-sample variation, normalisation of gene expression was performed against 18S ribosomal RNA. The genes used for normalisation should ideally not be subject to any regulation in the tissue or cells investigated (159).
When the gene expression analysis was performed, there were only a few gene sequences available for pigs. Far more gene sequences are currently available and it is now possible to purchase gene expression microarray that contain more than 20 000 pig genes.

Oxygen radical absorption capacity

The oxygen radical absorption capacity (ORAC) assay is a method for measuring the total antioxidant potential of a biological sample. The ORAC assay measures the time-dependent decrease in the fluorescence intensity of the β - PE (beta-phycoerythrin) marker protein resulting from its oxygen binding, and represents oxygen radical damage. On a molar basis, the β - PE protein reacts with oxygen radicals over 100 times slower than most biological antioxidants such as thiols, uric acid, bilirubin and ascorbate. However, β - PE is over 60 times more reactive than other non-antioxidant proteins. The analyser is programmed to record the fluorescence of β - PE every two minutes after the addition of AAPH (2, 2'-azobis (2-amidinopropane)dihydrochloride). Each reaction is calibrated using known standards of Trolox®, a water soluble vitamin E analogue. The results of the assay are reported on the basis of 1 ORAC unit = 1 μM Trolox® (172). The principal drawback of this assay is β - PE itself since it varies from lot to lot and is not very photostable (173). Considering these disadvantages an improved ORAC assay employing fluorescein (FL) as the fluorescent probe (ORAC-FL) was developed. FL as compared to β - PE does not interact with antioxidants, shows an excellent photostability,

and reduces the cost of experiments (174, 175). Unfortunately, this method was not yet established in the laboratory during this study but will be used in further projects.

Magnetic resonance imaging

Perfusion and diffusion magnetic resonance imaging (MRI) are two relatively new methods whereby tissue perfusion and cellular water mobility can be directly assessed (124). In perfusion MRI, the temporal effect of an iv injected contrast agent on the MR signal is measured. The observed effect can then be used to assess relative tissue perfusion by applying a kinetic model of the known distribution of the contrast agent in tissue. PWI has proved to be a sensitive method for detecting ischemia since the dynamic contrast agent effect directly reflects local tissue perfusion. In diffusion MRI, microscopic water diffusion can be measured by applying additional magnetic fields which vary as a function of time and positions. The resulting DWI image reflects the apparent diffusion coefficient (ADC) of water at the cellular level, which has proved to be an extremely sensitive marker for early cellular damage following HI. Both DWI and PWI suffer from limited spatial resolution compared with standard structural MRI. Therefore, both these techniques could only be used to assess functional changes affecting a large proportion of the cerebral volume in the present work. In further studies, a dedicated animal scanner with a smaller magnet bore and a higher field strength should be considered since this would improve the spatial resolution and signal-to-noise and thereby the sensitivity when detecting functional changes on a smaller scale. A suitable animal scanner was not available at Rikshospitalet when these studies were performed.

Summary of results

Paper I

Resuscitation with 100% O_2 Increases Cerebral Injury in Hypoxemic Piglets

Extracellular cerebral glycerol values were 50% higher in the piglets resuscitated with 100% oxygen compared to 21% oxygen at 90, 120 and 150 min after resuscitation ($p < 0.05$, independent sample t test at each time point). Total MMP activity was two-fold higher in resuscitated animals compared with controls ($p = 0.018$). MMP-2 activity was significantly higher in the animals resuscitated with 100% oxygen compared with 21% oxygen. mRNA expression of MMP-2 was significantly higher in piglets resuscitated with 100% oxygen compared with 21% oxygen ($p < 0.05$). Total antioxidant capacity, ORAC, was considerably lowered in the piglets resuscitated with 100% oxygen compared to 21% oxygen ($p = 0.001$). These results suggested a less favourable cerebral outcome in the group reoxygenated with 100% compared with 21% oxygen.

Mean arterial blood pressure, base excess and pH were all similar between the two resuscitation groups at baseline, at the end of the insult and at the end of the experiment.

Paper II

Resuscitation of hypoxic piglets with 100% oxygen increases pulmonary metalloproteinases and IL-8

In pulmonary tissue, pro- and active- MMP-2 levels were increased in piglets resuscitated with 100% O_2 compared to 21% O_2 ($p < 0.05$). Pro-MMP-9, total MMP activity and MMP-2 mRNA levels were significantly increased in resuscitated piglets compared to baseline ($p < 0.05$). Net gelatinolytic activity increased in submucosa and blood vessels after 100% O_2 ($p < 0.05$), and only in the blood vessels after 21% O_2. Compared to baseline, ORAC values were considerably lowered in the resuscitated piglets ($p < 0.05$) and significantly reduced in the 100% O_2 versus 21% O_2 group ($p < 0.05$). In BAL fluid, both pro-MMP-9 and pro-MMP-2 increased two-fold in the 100% O_2 group compared to 21% O_2 ($p < 0.05$). Moreover, IL-8 concentration increased significantly in piglets resuscitated with 100% O_2 compared to 21% O_2 ($p < 0.05$) suggesting a marked pro inflammatory response in the pulmonary tissue. Altogether, these data strongly suggest that caution must be taken when applying pure oxygen to the newborn infants.

Paper III

Increased myocardial matrix metalloproteinases in hypoxic newborn pigs during resuscitation: effects of oxygen and carbon dioxide

MMP-2 more than doubled from baseline values ($p < 0.001$), and was higher in piglets resuscitated with 100% O_2 than with ambient air ($p = 0.012$). The ORAC value was considerably decreased in piglets resuscitated with 100% O_2 compared with baseline ($p < 0.001$). In piglets with elevated $PaCO_2$, total MMP activity in the right ventricle was more increased than in the left ventricle ($p = 0.008$). In the left ventricle total MMP-activity was higher in piglets with low $PaCO_2$ than in piglets with elevated $PaCO_2$ ($p = 0.013$). In hypoxaemia-reoxygenation injury the MMP-2 level was highly increased and was most elevated in the piglets resuscitated with 100% O_2. Antioxidant capacity was considerably decreased. Assessed by total the MMP-activity, elevated $PaCO_2$ during resuscitation might protect the left ventricle, and probably increase the right ventricle injury of the myocardium.

Paper IV

Morphological and haemodynamic MR assessment of early neonatal brain injury in a piglet model

A linear correlation was observed between the relative cerebral perfusion reduction and the cerebral apparent diffusion coefficient (ADC) during HI ($r^2 = 0.85$, $p < 0.05$). There was no correlation between the rCBF reduction during 30 minutes of HI and the cerebral ADC after 30 or 150 minutes of reperfusion/reoxygenation (RR). The MR angiography enabled consistent assessment of the presence, absence and also recovery of complete occlusion of the extracranial carotid arteries. Ligation of the extracranial arteries in the piglet produced an unpredictable degree of ischemia due to considerable interindividual variations of vessel communication between the two hemispheres, between extra- and intracerebral arteries, and between vertebral and carotid arteries. A single bolus of intravascular contrast agent allowed measurement of perfusion and depiction of vessel anatomy, providing a comprehensive tool for both a morphological and haemodynamic assessment of ischemia and hypoxia.

Discussion

Our findings demonstrate that resuscitation of hypoxic piglets with 100% O_2 causes more upregulation of early markers of tissue injury than resuscitation with 21% O_2. This was detected in cerebral, pulmonary as well as myocardial tissue based on extracellular markers of tissue damage and matrix metalloproteinases. The time span of these experiments is relatively short. After hypoxia and resuscitation, the piglets were observed for only two and a half hours. We are therefore not able to conclude whether the observed changes are relevant for the long-term outcome. However, the acute tissue damage markers glycerol (in brain) and IL-8 (in lung) were considerably increased – allowing us to believe that the tissue damage in the piglets is significant and would probably have affected their clinical outcome. It is interesting that the MMPs were significantly increased in the brain, heart, and lungs when resuscitated with pure oxygen.

There is an ongoing debate on whether to use ambient air or 100% O_2 in neonatal resuscitation (44, 176-178). Clinical studies have even indicated that hyperoxic treatment of the newborn may have detrimental effects in childhood. For term and near-term infants, Davis et al conclude that air should be used initially, with oxygen as backup if initial resuscitation fails (53)**.** A Cochrane review concludes that there is insufficient evidence at present on whether to recommend room air or 100% oxygen for newborn resuscitation. If ambient air is chosen as the initial gas for resuscitation, supplementary oxygen should be available (179).

Our findings, showing the detrimental effects of hyperoxic resuscitation in the piglet, add information to this debate. Further studies must be done to investigate mechanisms that

explain our findings. Gene activation, repair and down-regulation in different tissue, following global hypoxia and hyperoxic resuscitation should be analysed. It seems that pure oxygen in contrast to room air for resuscitation changes a number of genes and that a similar effect is found in multiple organs in the piglet. Secondary energy failure has been described in the brain after hypoxia- ischemia (21). The effect of hyperoxia on mitochondrial function should be investigated by determining the levels of electron transport chain proteins encoded by mitochondrial and nuclear DNA, assessing the effect of hyperoxia on oxidative phosphorylation and the transcription of nuclear and mitochondrial genes.

HIE remains a major marker of perinatal and neonatal morbidity, as well as of permanent neurodevelopmental disability. Early detection is crucial for interventions aimed at preventing or reversing ongoing injury. Currently, treatment of HIE lacks accurate predictors for the long-term outcome (6). We conclude that MRI is capable of detecting early cerebral changes after hypoxia-ischemia with diffusion and perfusion MRI. This is a promising and powerful tool regarding the utility of MRI to monitor early cerebral damage, although we do not know at the moment the ideal time span for MRI investigations in the newborn. In addition, proton magnetic resonance spectroscopy (HMRS) will enable us to measure the various metabolite ratios *in vivo* in normal and pathologic conditions (6). The combination of MRI and HMRS may make it possible to evaluate severity and may help predict the prognosis of HIE.

This thesis has investigated the acute changes after hypoxia-reperfusion. Further animal and clinical studies must be done to assess the long-term effects.

Like every other drug, oxygen also has side-effects. However, oxygen will always be an important drug in medicine. Oxygen supply should be optimised regarding dosage, concentration and time. If oxygen is given, it should be tapered as quickly as possible. Optimally, oxygen saturation should be followed continuously by a pulse oxymeter. The recommended dosage of oxygen during newborn resuscitation will most likely be reduced.

Conclusions

I Cerebral, pulmonary and myocardial tissue damage was more extensive assessed by MMPs in the group resuscitated with 100% O_2 compared with 21% O_2 suggesting a less favourable outcome with pure oxygen (Paper I, II, and III).

II Cerebral extracellular glycerol and pulmonary IL-8 was more extensive in the group resuscitated with 100% O_2 compared with 21% O_2. This suggests a less favourable cerebral and pulmonary outcome in the hyperoxic group (Paper I and II).

III There were no significant differences in any cerebral or pulmonary outcome measures between different $PaCO_2$ levels (Paper I and II).

IV Elevated $PaCO_2$ during resuscitation might protect the left ventricle, and probably increase right ventricle damage of the myocardium. The resuscitation procedure is

important to the myocardium since the $PaCO_2$ level also has to be addressed to avoid additional iatrogenic tissue injury during the resuscitation of neonatal piglets (Paper III).

V Diffusion and perfusion MR imaging enabled consistent detection of early cerebral changes, and MR angiography provided visualization of the presence, absence, and recovery of complete carotid occlusion (Paper IV).

References

1. Saugstad OD, Aasen AO 1980 Plasma hypoxanthine concentrations in pigs. A prognostic aid in hypoxia. Eur Surg Res 12:123-129

2. UNICEF. Child Mortality Statistics. 2003. Ref Type: Report

3. Volpe J 2001 Neurology of the newborn. W.B.Saunders Company, Philadelphia,

4. Low JA 1997 Intrapartum fetal asphyxia: definition, diagnosis, and classification. Am J Obstet Gynecol 176:957-959

5. American College of Obstetricians and Gynecologists Committee on Obstetric Practice 2004 ACOG Committee Opinion #303: Inappropriate use of the terms fetal distress and birth asphyxia. Obstet Gynecol 104:903-904

6. Fan G, Wu Z, Chen L, Guo Q, Ye B, Mao J 2003 Hypoxia-ischemic encephalopathy in full-term neonate: correlation proton MR spectroscopy with MR imaging. Eur J Radiol 45:91-98

7. Nelson KB 2003 'Defining hypoxic-ischemic birth events'. Dev Med Child Neurol 45:71

8. Tapia-Rombo CA, Carpio-Hernandez JC, Salazar-Acuna AH, Alvarez-Vazquez E, Mendoza-Zanella RM, Perez-Olea V, Rosas-Fernandez C 2000 Detection of transitory myocardial ischemia secondary to perinatal asphyxia. Arch Med Res 31:377-383

9. Leuthner SR, Das UG 2004 Low Apgar scores and the definition of birth asphyxia. Pediatr Clin North Am 51:737-745

10. Sarnat H, Sarnat M 1976 Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. Arch Neurol 33:696-705

11. Levene ML, Kornberg J, Williams TH 1985 The incidence and severity of post-asphyxial encephalopathy in full-term infants. Early Hum Dev 11:21-26

12. Yu VY 1994 Prognosis in infants with birth asphyxia. Chung Hua Min Kuo Hsiao Erh Ko I Hsueh Hui Tsa Chih 35:481-486

13. Hermansen MC 2003 The acidosis paradox: asphyxial brain injury without coincident acidemia. Dev Med Child Neurol 45:353-356

14. Downing SE, Talner NS, Gardner TH 1966 Influences of hypoxemia and acidemia on left ventricular function. Am J Physiol 210:1327-1334

15. Andres RL, Saade G, Gilstrap LC, Wilkins I, Witlin A, Zlatnik F, Hankins GV 1999 Association between umbilical blood gas parameters and neonatal morbidity and death in neonates with pathologic fetal acidemia. Am J Obstet Gynecol 181:867-871

16. Pasternak JF 1993 Hypoxic-ischemic brain damage in the term infant. Lessons from the laboratory. Pediatr Clin North Am 40:1061-1072

17. Ranjit MS 2000 Cardiac abnormalities in birth asphyxia. Indian J Pediatr 67:26-29

18. Volpe JJ 2001 Perinatal brain injury: from pathogenesis to neuroprotection. Ment Retard Dev Disabil Res Rev 7:56-64

19. Fellman V, Raivio KO 1997 Reperfusion injury as the mechanism of brain damage after perinatal asphyxia. Pediatr Res 41:599-606

20. Berger R, Garnier Y, Jensen A 2002 Perinatal brain damage: underlying mechanisms and neuroprotective strategies. J Soc Gynecol Investig 9:319-328

21. Siesjo BK, Siesjo P 1996 Mechanisms of secondary brain injury. Eur J Anaesthesiol 13:247-268

22. Gluckman PD, Wyatt JS, Azzopardi D, Ballard R, Edwards AD, Ferriero DM, Polin RA, Robertson CM, Thoresen M, Thoresen M, Whitelaw A, Gunn AJ 2005 Head cooling in neonatal hypoxic-ischaemic encephalopathy. Lancet 365:663-670

23. Gluckman PD, Wyatt JS, Azzopardi D, Ballard R, Edwards AD, Ferriero DM, Polin RA, Robertson CM, Thoresen M, Whitelaw A, Gunn AJ 2005 Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. Lancet 365:663-670

24. Nelson KB, Grether JK 1995 Can magnesium sulfate reduce the risk of cerebral palsy in very low birthweight infants? Pediatrics 95:263-269

25. Garnier Y, Middelanis J, Jensen A, Berger R 2002 Neuroprotective effects of magnesium on metabolic disturbances in fetal hippocampal slices after oxygen-

glucose deprivation: mediation by nitric oxide system. J Soc Gynecol Investig 9:86-92

26. Jensen A, Garnier Y, Middelanis J, Berger R 2003 Perinatal brain damage--from pathophysiology to prevention. Eur J Obstet Gynecol Reprod Biol 110:70-79

27. May C, Greenough A 2004 Corticosteroids in infant chronic lung disease. Monaldi Arch Chest Dis 61:162-166

28. Lemons JA, Bauer CR, Oh W, Korones SB, Papile LA, Stoll BJ, Verter J, Temprosa M, Wright LL, Ehrenkranz RA, Fanaroff AA, Stark A, Carlo W, Tyson JE, Donovan EF, Shankaran S, Stevenson DK 2001 Very low birth weight outcomes of the National Institute of Child health and human development neonatal research network, January 1995 through December 1996. NICHD Neonatal Research Network. Pediatrics 107:1-8

29. Kotecha S, Silverman M 1999 Chronic respiratory complications of neonatal disorders. In: Landau LI, Taussig L, Mosby (eds) Textbook of pediatric respiratory medicine. pp 499-521

30. Halliday HL, Ehrenkranz RA, Doyle LW 2003 Early postnatal (<96 hours) corticosteroids for preventing chronic lung disease in preterm infants. [Update of Cochrane Database Syst Rev. 2001 ;(1):CD001146; PMID: 11279706]. [Review] [53 refs]. CDS Rev 1:CD001146, C

31. Jobe AH, Ikegami M 2001 Antenatal infection/inflammation and postnatal lung maturation and injury. Respir Res 2:27-32

32. Bustani P, Kotecha S 2003 Role of cytokines in hyperoxia mediated inflammation in the developing lung. Front Biosci 8:694-704

33. Speer CP 2004 Pre- and postnatal inflammatory mechanisms in chronic lung disease of preterm infants. Paediatr Respir Rev 5:241-244

34. Sweet DG, McMahon KJ, Curley AE, O'Connor CM, Halliday HL 2001 Type I collagenases in bronchoalveolar lavage fluid from preterm babies at risk of developing chronic lung disease. Arch Dis Child Fetal Neonatal Ed 84:F168-F171

35. Sweet DG, Curley AE, Chesshyre E, Pizzotti J, Wilbourn MS, Halliday HL, Warner JA 2004 The role of matrix metalloproteinases-9 and -2 in development of neonatal chronic lung disease. Acta Paediatr 93:791-796

36. Rowe RD, Izukawa T, Mulholland HC, Bloom KR, Cook DH, Swyer PR 1978 Nonstructural heart disease in the newborn. Observations during one year in a perinatal service. Arch Dis Child 53:726-730

37. Hambraeus-Jonzon K, Bindslev L, Mellgard AJ, Hedenstierna G 1997 Hypoxic pulmonary vasoconstriction in human lungs. A stimulus-response study. Anesthesiology 86:308-315

38. Hirsch R, Landt Y, Porter S, Canter CE, Jaffe AS, Ladenson JH, Grant JW, Landt M 1997 Cardiac troponin I in pediatrics: normal values and potential use in the assessment of cardiac injury. J Pediatr 130:872-877

39. Borke WB, Munkeby BH, Morkrid L, Thaulow E, Saugstad OD 2004 Resuscitation with 100% O2 does not protect the myocardium in hypoxic newborn piglets. Arch Dis Child Fetal Neonatal Ed 89:F156-F160

40. Apgar V 1953 A proposal for a new method of evaluation of the newborn infant. Curr Res Anesth Analg260-267

41. Ruth VJ, Raivio KO 1988 Perinatal brain damage: predictive value of metabolic acidosis and the Apgar score. BMJ 297(6640):24-7,

42. Seidman DS, Paz I, Laor A, Gale R, Stevenson DK, Danon YL 1991 Apgar scores and cognitive performance at 17 years of age. Obstet Gynecol 77:875-878

43. Casey BM, McIntire DD, Leveno KJ 2001 The continuing value of the Apgar score for the assessment of newborn infants. N Engl J Med 344:467-471

44. Niermeyer S, Vento M 2004 Is 100% oxygen necessary for the resuscitation of newborn infants? J Matern Fetal Neonatal Med 15:75-84

45. Howell M, Ford P 1985 The paradoxes of a small American disaster. In: Howell M, Ford P (eds) The Beetle of Aphrodite. Random House, New York, pp 210-236

46. Saugstad OD 2002 Oxygen Supplementation in the Newborn Period: Do We Know the Consequences? In: Fanaroff A MMSD (ed) Yearbook of neonatal and perinatal medicine. Mosby, Chicago, pp XV-XXII

47. Silverman WA 2004 A cautionary tale about supplemental oxygen: the albatross of neonatal medicine. Pediatrics 113:394-396

48. The STOP-ROP Multicenter Study Group 2000 Supplemental Therapeutic Oxygen for Prethreshold Retinopathy of Prematurity (STOP-ROP), A Randomized, Controlled Trial. I: Primary Outcomes. Pediatrics 105:295-310

49. Ramji S, Ahuja S, Thirupuram S, Rootwelt T, Rooth G, Saugstad OD 1993 Resuscitation of asphyxic newborn infants with room air or 100% oxygen. Pediatr Res 34:809-812

50. Saugstad OD, Rootwelt T, Aalen O 1998 Resuscitation of asphyxiated newborn infants with room air or oxygen: an international controlled trial: the Resair 2 study. Pediatrics 102:e1

51. Vento M, Asensi M, Sastre J, Garcia-Sala F, Pallardo FV, Vina J 2001 Resuscitation with room air instead of 100% oxygen prevents oxidative stress in moderately asphyxiated term neonates. Pediatrics 107:642-647

52. Temesvari P, Karg E, Bodi I, Nemeth I, Pinter S, Lazics K, Domoki F, Bari F 2001 Impaired early neurologic outcome in newborn piglets reoxygenated with 100% oxygen compared with room air after pneumothorax-induced asphyxia. Pediatr Res 49:812-819

53. Davis PG, Tan A, O'Donnell CP, Schulze A 2004 Resuscitation of newborn infants with 100% oxygen or air: a systematic review and meta-analysis. Lancet 364:1329-1333

54. Saugstad O, Ramji S, Vento M 2005 Resuscitation of Depressed Newborn Infants with Ambient Air or Pure Oxygen: A Meta-Analysis. Biol Neonate 87:27-34

55. Contributors and Reviewers for the Neonatal Resuscitation Guidelines 2000 International Guidelines for Neonatal Resuscitation: An Excerpt From the Guidelines 2000 for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care: International Consensus on Science. Pediatrics 106:e29

56. Wiswell TE 2003 Neonatal resuscitation. Respir Care 48:288-294

57. Tin W 2004 Optimal Oxygen Saturation for Preterm Babies. Biol Neonate 85:319-325

58. Contributors and Reviewers for the Neonatal Resuscitation Guidelines 2000 International Guidelines for Neonatal Resuscitation: An Excerpt From the Guidelines 2000 for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care: International Consensus on Science. Pediatrics 106:e29

59. Steiner H, Neligan G 1975 Perinatal cardiac arrest. Quality of the survivors. Arch Dis Child 50:696-702

60. Scott H 1976 Outcome of very severe birth asphyxia. Arch Dis Child 51:712-716

61. Thomson AJ, Searle M, Russell G 1977 Quality of survival after severe birth asphyxia. Arch Dis Child 52:620-626

62. Jain L, Ferre C, Vidyasagar D, Nath S, Sheftel D 1991 Cardiopulmonary resuscitation of apparently stillborn infants: survival and long-term outcome. J Pediatr 118:778-782

63. Gerschman R, Gilbert DL, Nye SW, Dwyer P, Fenn WO 1954 Oxygen poisoning and x-irradiation: a mechanism in common. Science 119:623-626

64. Haase E, Bigam DL, Nakonechny QB, Jewell LD, Korbutt G, Cheung PY 2004 Resuscitation with 100% oxygen causes intestinal glutathione oxidation and reoxygenation injury in asphyxiated newborn piglets. Annals of Surgery 240(2):364-73,

65. Pitkanen OM, Hallman M, Andersson SM 1990 Correlation of free oxygen radical-induced lipid peroxidation with outcome in very low birth weight infants. J Pediatr 116:760-764

66. Halliwell B 1992 Reactive oxygen species and the central nervous system. J Neurochem 59:1609-1623

67. Hjort MR, Brenyo AJ, Finkelstein JN, Frampton MW, LoMonaco MB, Stewart JC, Johnston CJ, D'Angio CT 2003 Alveolar epithelial cell-macrophage interactions affect oxygen-stimulated interleukin-8 release. Inflammation 27:137-145

68. Robertson N 2005 Air or 100% oxygen for asphyxiated babies? Time to decide. Crit Care 9:128-130

69. Hansbrough F, Priebe CJ, Jr., Falterman KW, Bornside GH, Welsh RA 1983 Pathogenesis of early necrotizing enterocolitis in the hypoxic neonatal dog. Am J Surg 145:169-175

70. Saugstad OD 1988 Hypoxanthine as an indicator of hypoxia: its role in health and disease through free radical production. Pediatr Res 23:143-150

71. Gladstone IM Jr, LR 1994 Oxidation of proteins in neonatal lungs. Pediatrics 93:764-768

72. Varsila E, Pitkanen O, Hallman M, Andersson S 1994 Immaturity-dependent free radical activity in premature infants. Pediatr Res 36:55-59

73. Hammerman C, Kaplan M 1998 Ischemia and reperfusion injury. The ultimate pathophysiologic paradox. Clin Perinatol 25:757-777

74. Phelps DL 1992 Retinopathy of prematurity. Curr Probl Pediatr 22:349-371

75. Saugstad OD 1998 Chronic lung disease: the role of oxidative stress. Biol Neonate 74: Suppl-8

76. Volpe JJ 1997 Brain injury in the premature infant--from pathogenesis to prevention. Brain Dev519-534

77. Inder TE, Volpe JJ 2000 Mechanisms of perinatal brain injury. Semin Neonatol 5:3-16

78. Sabatino GM, Domizio S, Cicioni P, Sabatino G 2003 Mechanisms of perinatal brain injury. Panminerva Med 45:117-121

79. Haase E, Bigam DL, Nakonechny QB, Rayner D, KG, Cheung PY 2005 Cardiac function, myocardial glutathione, and matrix metalloproteinase-2 levels in

hypoxic newborn pigs reoxygenated by 21%, 50%, or 100% oxygen. Shock 23:383-389

80. Weinberger B, Laskin DL, Heck DE, Laskin JD 2002 Oxygen toxicity in premature infants. Toxicol Appl Pharmacol 181:60-67

81. Ferriero DM 2004 Neonatal Brain Injury. N Engl J Med 351:1985-1995

82. Masters CJ 1996 Cellular signalling: the role of the peroxisome. Cell Signal 8:197-208

83. Saugstad OD, Sanderud J 1989 Circulatory effects of oxygen radicals. Biomed Biochim Acta 48:20-24

84. Iida Y, Katusic ZS 2000 Mechanisms of cerebral arterial relaxations to hydrogen peroxide. Stroke 31:2224-2230

85. Vanhoutte PM 2001 Endothelium-derived free radicals: for worse and for better. J Clin Invest 107:23-25

86. Tse HM, Milton MJ, Piganelli JD 2004 Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: implication for their use in targeting oxidation-reduction reactions in innate immunity. Free Radic Biol Med 36:233-247

87. Zhang HJ, Zhao W, Venkataraman S, Robbins ME, Buettner GR, Kregel KC, Oberley LW 2002 Activation of matrix metalloproteinase-2 by overexpression of

manganese superoxide dismutase in human breast cancer MCF-7 cells involves reactive oxygen species. J Biol Chem 277:20919-20926

88. Okamoto T, Akaike T, Nagano T, Miyajima S, Suga M, Ando M, Ichimori K, Maeda H 1997 Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide. Arch Biochem Biophys 342:261-274

89. Rosenberg GA, Estrada EY, Dencoff JE 1998 Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain. Stroke 29:2189-2195

90. Gushima Y, Ichikado K, Suga M, Okamoto T, Iyonaga K, Sato K, Miyakawa H, Ando M 2001 Expression of matrix metalloproteinases in pigs with hyperoxia-induced acute lung injury. Eur Respir J 18:827-837

91. Rivera S, Ogier C, Jourquin J, Timsit S, Szklarczyk AW, Miller K, Gearing AJ, Kaczmarek L, Khrestchatisky M 2002 Gelatinase B and TIMP-1 are regulated in a cell- and time-dependent manner in association with neuronal death and glial reactivity after global forebrain ischemia. Eur J Neurosci 15:19-32

92. Wang WM, Schulze CJM, Suarez-Pinzon WLM, Dyck JRB, Sawicki GP, Schulz RP 2002 Intracellular Action of Matrix Metalloproteinase-2 Accounts for Acute Myocardial Ischemia and Reperfusion Injury. Circulation 106:1543-1549

93. Ben Yosef Y, Lahat N, Shapiro S, Bitterman H, Miller A 2002 Regulation of endothelial matrix metalloproteinase-2 by hypoxia/reoxygenation. Circ Res 90:784-791

94. Singh RB, Dandekar SP, Elimban V, Gupta SK, Dhalla NS 2004 Role of proteases in the pathophysiology of cardiac disease. Mol Cell Biochem 263:241-256

95. Jourquin J, Tremblay E, Decanis N, Charton G, Hanessian S, Chollet AM, Le Diguardher T, Khrestchatisky M, Rivera S 2003 Neuronal activity-dependent increase of net matrix metalloproteinase activity is associated with MMP-9 neurotoxicity after kainate. Eur J Neurosci 18:1507-1517

96. Woessner JF, Jr. 1991 Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 5:2145-2154

97. Brew K, Dinakarpandian D, Nagase H 2000 Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta 1477:267-283

98. Cederqvist K, Sorsa T, Tervahartiala T, Maisi P, Reunanen K, Lassus P, Andersson S 2001 Matrix Metalloproteinases-2, -8, and -9 and TIMP-2 in Tracheal Aspirates From Preterm Infants With Respiratory Distress. Pediatrics 108:686-692

99. Schock BC, Sweet DG, Ennis M, Warner JA, Young IS, Halliday HL 2001 Oxidative stress and increased type-IV collagenase levels in bronchoalveolar lavage fluid from newborn babies. Pediatr Res 50:29-33

100. Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, Lipton SA 2002 S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. Science 297:1186-1190

101. Gasche Y, Copin JC, Sugawara T, Fujimura M, Chan PH 2001 Matrix metalloproteinase inhibition prevents oxidative stress-associated blood-brain barrier disruption after transient focal cerebral ischemia. J Cereb Blood Flow Metab 21:1393-1400

102. Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW, Schulz R 2000 Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. Circulation 101:1833-1839

103. Kameda K, Matsunaga T, Abe N, Hanada H, Ishizaka H, Ono H, Saitoh M, Fukui K, Fukuda I, Osanai T, Okumura K 2003 Correlation of oxidative stress with activity of matrix metalloproteinase in patients with coronary artery disease. Possible role for left ventricular remodelling. Eur Heart J 24:2180-2185

104. Lalu MM, Pasini E, Schulze CJ, Ferrari-Vivaldi M, Ferrari-Vivaldi G, Bachetti T, Schulz R 2005 Ischaemia-reperfusion injury activates matrix metalloproteinases in the human heart. Eur Heart J 26:27-35

105. Marklund N, Salci K, Lewen A, Hillered L 1997 Glycerol as a marker for post-traumatic membrane phospholipid degradation in rat brain. Neuroreport 8:1457-1461

106. Hillered L, Valtysson J, Enblad P, Persson L 1998 Interstitial glycerol as a marker for membrane phospholipid degradation in the acutely injured human brain. J Neurol Neurosurg Psychiatry 64:486-491

107. Frykholm P, Hillered L, Langstrom B, Persson L, Valtysson J, Watanabe Y, Enblad P 2001 Increase of interstitial glycerol reflects the degree of ischaemic brain damage: a PET and microdialysis study in a middle cerebral artery occlusion-reperfusion primate model. J Neurol Neurosurg Psychiatry 71:455-461

108. Martin LJ, Brambrink AM, Price AC, Kaiser A, Agnew DM, Ichord RN, Traystman RJ 2000 Neuronal death in newborn striatum after hypoxia-ischemia is necrosis and evolves with oxidative stress. Neurobiol Dis 7:169-191

109. Li A, Dubey S, Varney ML, Dave BJ, Singh RK 2003 IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. J Immunol 170:3369-3376

110. D'Angio CT, LoMonaco MB, Chaudhry SA, Paxhia A, Ryan RM 1999 Discordant pulmonary proinflammatory cytokine expression during acute hyperoxia in the newborn rabbit. Exp Lung Res 25:443-465

111. Groneck P, Gotze-Speer B, Oppermann M, Eiffert H, Speer CP 1994 Association of pulmonary inflammation and increased microvascular permeability during the

development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluids of high-risk preterm neonates. Pediatrics 93:712-718

112. Speer CP 2001 New insights into the pathogenesis of pulmonary inflammation in preterm infants. Biol Neonate 79:205-209

113. Kotecha S, Chan B, Azam N, Silverman M, Shaw RJ 1995 Increase in interleukin-8 and soluble intercellular adhesion molecule-1 in bronchoalveolar lavage fluid from premature infants who develop chronic lung disease. Arch Dis Child Fetal Neonatal Ed 72:F90-F96

114. Baier RJ, Loggins J, Kruger TE 2001 Monocyte chemoattractant protein-1 and interleukin-8 are increased in bronchopulmonary dysplasia: relation to isolation of Ureaplasma urealyticum. J Investig Med 49:362-369

115. Munshi UK, Niu JO, Siddiq MM, Parton LA 1997 Elevation of interleukin-8 and interleukin-6 precedes the influx of neutrophils in tracheal aspirates from preterm infants who develop bronchopulmonary dysplasia. Pediatr Pulmonol 24:331-336

116. Golej J, Winter P, Schoffmann G, Kahlbacher H, Stoll E, Boigner H, Trittenwein G 2002 Impact of extracorporeal membrane oxygenation modality on cytokine release during rescue from infant hypoxia. Shock 20:110-115

117. Huppi PS, Inder TE 2001 Magnetic resonance techniques in the evaluation of the perinatal brain: recent advances and future directions. Semin Neonatol 6:195-210

118. D'Arceuil HE, de Crespigny AJ, Rother J, Seri S, Moseley ME, Stevenson DK, Rhine W 1998 Diffusion and perfusion magnetic resonance imaging of the evolution of hypoxic ischemic encephalopathy in the neonatal rabbit. J Magn Reson Imaging 8:820-828

119. Shih LC, Saver JL, Alger JR, Starkman S, Leary MC, Vinuela F, Duckwiler G, Gobin YP, Jahan R, Villablanca JP, Vespa PM, Kidwell CS 2003 Perfusion-weighted magnetic resonance imaging thresholds identifying core, irreversibly infarcted tissue. Stroke 34:1425-1430

120. Huisman AGM, Gregory S 2004 Perfusion-weighted magnetic resonance imaging of the brain: techniques and application in children. Eur Radiol 14:59-72

121. Vermeulen RJ, Fetter WP, Hendrikx L, Van Schie PE, Van Der Knaap MS, Barkhof F 2003 Diffusion-weighted MRI in severe neonatal hypoxic ischaemia: the white cerebrum. Neuropediatrics 34:72-76

122. Moseley ME, Cohen Y, Mintorovitch J, Chileuitt L, Shimizu H, Kucharczyk J, Wendland MF, Weinstein PR 1990 Early detection of regional cerebral ischemia in cats: comparison of diffusion- and T2-weighted MRI and spectroscopy. Magn Reson Med 14:330-346

123. Le Bihan D, Mangin JF, Poupon C, Clark CA, Pappata S, Molko N, Chabriat H 2001 Diffusion tensor imaging: concepts and applications. J Magn Reson Imaging 13:534-546

124. Rutherford M, Ward P, Allsop J, Malamatentiou C, Counsell S 2005 Magnetic resonance imaging in neonatal encephalopathy. Early Hum Dev 81:13-25

125. Huppi PS, Murphy B, Maier SE, Zientara GP, Inder TE, Barnes PD, Kikinis R, Jolesz FA, Volpe JJ 2001 Microstructural brain development after perinatal cerebral white matter injury assessed by diffusion tensor magnetic resonance imaging. Pediatrics 107:455-460

126. Donnan GA, Davis SM 2002 Neuroimaging, the ischaemic penumbra, and selection of patients for acute stroke therapy. Lancet Neurol 1:417-425

127. Egeli AK, Framstad T, Gronningen D 1998 The effect of peroral administration of amino acid-chelated iron to pregnant sows in preventing sow and piglet anaemia. Acta Vet Scand 39:77-87

128. Myers RE 1972 Two patterns of perinatal brain damage and their conditions of occurrence. Am J Obstet Gynecol 112:246-276

129. Myers R.E 1977 Experimental Models of Perinatal Brain Damage: Relevance to Human Pathology. In: Gluck L (ed) Intrauterine Asphyxia and the Developing Fetal Brain. Year Book Medical Publishers, Inc., 1977. Chicago, pp 37-97

130. Hannon JP, Bossone CA, Wade CE 1990 Normal physiological values for conscious pigs used in biomedical research. Lab Anim Sci 40:293-298

131. Raju TN 1992 Some animal models for the study of perinatal asphyxia. Biol Neonate 62:202-214

132. Roohey T, Raju TN, Moustogiannis AN 1997 Animal models for the study of perinatal hypoxic-ischemic encephalopathy: a critical analysis. Early Hum Dev 47:115-146

133. Swindle MM, Smith AC, Hepburn BJ 1988 Swine as models in experimental surgery. J Invest Surg 1:65-79

134. Tumbleson M 1986 Swine in biomedical research. Plenum Press, New York,

135. Pond WG, Boleman SL, Fiorotto ML, Ho H, Knabe DA, Mersmann HJ, Savell JW, Su DR 2000 Perinatal ontogeny of brain growth in the domestic pig. Proc Soc Exp Biol Med 223:102-108

136. Dickerson JWT, Dobbing J 1967 Prenatal and postnatal growth and development of the central nervous system of the pig. Proc R Soc Lond B Biol Sci 166:384-395

137. Grate LL, Golden JA, Hoopes PJ, Hunter JV, Duhaime AC 2003 Traumatic brain injury in piglets of different ages: techniques for lesion analysis using histology and magnetic resonance imaging. J Neurosci Methods 123:201-206

138. Laptook A, Stonestreet BS, Oh W 1982 The effects of different rates of plasmanate infusions upon brain blood flow after asphyxia and hypotension in newborn piglets. J Pediatr 100:791-796

139. Harada J, Takaku A, Endo S, Kuwayama N, Fukuda O 1991 Differences in critical cerebral blood flow with age in swine. J Neurosurg 75:103-107

140. Haworth SG, Hislop AA 1981 Adaptation of the pulmonary circulation to extra-uterine life in the pig and its relevance to the human infant. Cardiovasc Res 15:108-119

141. Barnes RJ, Comline RS, Dobson A, Silver M, Burton GJ, Steven DH 1979 On the presence of a ductus venosus in the fetal pig in late gestation. J Dev Physiol 1:105-110

142. Miller R 2000 Anesthesia. Churchill Livingston, New York,

143. Rajan V, Beharry KD, Williams P, Modanlou HD 1998 Pharmacodynamic effects and pharmacokinetic profile of continuous infusion fentanyl in newborn piglets. Biol Neonate 74:39-47

144. Steffey EP, Baggot JD, Eisele JH, Willits N, Woliner MJ, Jarvis KA, Elliott AR, Tagawa M 1994 Morphine-isoflurane interaction in dogs, swine and rhesus monkeys. J Vet Pharmacol Ther 17:202-210

145. Nussmeier NA, Benthuysen JL, Steffey EP, Anderson JH, Carstens EE, Eisele JH, Jr., Stanley TH 1991 Cardiovascular, respiratory, and analgesic effects of fentanyl in unanesthetized rhesus monkeys. Anesth Analg 72:221-226

146. Maurer R 1982 Multiplicity of opiate receptors in different species. Neurosci Lett 30:303-307

147. Moon PF, Scarlett JM, Ludders JW, Conway TA, Lamb SV 1995 Effect of fentanyl on the minimum alveolar concentration of isoflurane in swine. Anesthesiology 83:535-542

148. Steffey EP 1995 Isoflurane-sparing effect of fentanyl in swine. Relevance and importance. Anesthesiology 83:446-448

149. Goodman L, Gilman A 1971 The Pharmacological Basis of Therapeutics. The Macmillian Company, New York,

150. Strom J, Haggmark S, Reiz S, Sorensen MB 1987 Cardiovascular effects of pentobarbital in pigs, and the lack of response to naloxone in pentobarbital induced circulatory failure. Acta Anaesthesiol Scand 31:413-416

151. Thurmon J, Tranquilli W 1986 Anaesthesia for cardiovascular research. In: Stanton H, Mersmann H (eds) Swine in cardiovascular research. CRP Press Inc., Boca Raton, Florida, pp 39-60

152. Worek FS, Blumel G, Zeravik J, Zimmermann GJ, Pfeiffer UJ 1988 Comparison of ketamine and pentobarbital anesthesia with the conscious state in a porcine model of Pseudomonas aeruginosa septicemia. Acta Anaesthesiol Scand 32:509-515

153. Softeland E, Framstad T, Thorsen T, Holmsen H 1995 Evaluation of thiopentone-midazolam-fentanyl anaesthesia in pigs. Lab Anim 29:269-275

154. Steen PA, Milde JH, Michenfelder JD 1978 Cerebral metabolic and vascular effects of barbiturate therapy following complete global ischemia. J Neurochem 31:1317-1324

155. Albrecht RF, Miletich DJ, Rosenberg R, Zahed B 1977 Cerebral blood flow and metabolic changes from induction to onset of anesthesia with halothane or pentobarbital. Anesthesiology 47:252-256

156. Smith AL, Hoff JT, Nielsen SL, Larson CP 1974 Barbiturate protection in acute focal cerebral ischemia. Stroke 5:1-7

157. Araki T, Kato H, Kogure K, Inoue T 1990 Regional neuroprotective effects of pentobarbital on ischemia-induced brain damage. Brain Res Bull 25:861-865

158. Smith AC, Zellner JL, Spinale FG, Swindle MM 1991 Sedative and cardiovascular effects of midazolam in swine. Lab Anim Sci 41:157-161

159. Ahmad R, Beharry K, Modanlou H 2000 Changes in cerebral venous prostanoids during midazolam-induced cerebrovascular hypotension in newborn piglets. Crit Care Med 28:2429-2436

160. Swindle MM, Horneffer PJ, Gardner TJ, Gott VL, Hall TS, Stuart RS, Baumgartner WA, Borkon AM, Galloway E, Reitz BA 1986 Anatomic and anesthetic considerations in experimental cardiopulmonary surgery in swine. Lab Anim Sci 36:357-361

161. Ungerstedt U 1991 Microdialysis--principles and applications for studies in animals and man. J Intern Med 230:365-373

162. Menzel M, Doppenberg EM, Zauner A, Soukup J, Reinert MM, Clausen T, Brockenbrough PB, Bullock R 1999 Cerebral oxygenation in patients after severe head injury: monitoring and effects of arterial hyperoxia on cerebral blood flow, metabolism and intracranial pressure. J Neurosurg Anesthesiol 11:240-251

163. Hutchinson PJ, Al Rawi PG, O'Connell MT, Gupta AK, Maskell LB, Hutchinson DB, Pickard JD, Kirkpatrick PJ 1999 Monitoring of brain metabolism during aneurysm surgery using microdialysis and brain multiparameter sensors. Neurol Res 21:352-358

164. Peerdeman SM, Girbes AR, Vandertop WP 2000 Cerebral microdialysis as a new tool for neurometabolic monitoring. Intensive Care Med 26:662-669

165. Peerdeman SM, Girbes AR, Polderman KH, Vandertop WP 2003 Changes in cerebral interstitial glycerol concentration in head-injured patients; correlation with secondary events. Intensive Care Med 29:1825-1828

166. Woessner JF, Jr. 1995 Quantification of matrix metalloproteinases in tissue samples. Methods Enzymol 248:510-528

167. Kleiner DE, Stetler-Stevenson WG 1994 Quantitative zymography: detection of picogram quantities of gelatinases. Anal Biochem 218:325-329

168. Yan SJ, Blomme EAG 2003 In Situ Zymography: A Molecular Pathology Technique to Localize Endogenous Protease Activity in Tissue Sections. Vet Pathol 40:227-236

169. Frederiks WM, Mook OR 2004 Metabolic mapping of proteinase activity with emphasis on in situ zymography of gelatinases: review and protocols. J Histochem Cytochem 52:711-722

170. Snoek-van Beurden PA VdHJW 2005 Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. Biotechniques 38:78-83

171. Galis ZS, Sukhova GK, Libby P 1995 Microscopic localization of active proteases by in situ zymography: detection of matrix metalloproteinase activity in vascular tissue. FASEB J 9:974-980

172. Cao G, Alessio HM, Cutler RG 1993 Oxygen-radical absorbance capacity assay for antioxidants. Free Radic Biol Med 14:303-311

173. Ou B, Hampsch-Woodill M, Prior RL 2001 Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J Agric Food Chem 49:4619-4626

174. Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL 2002 High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J Agric Food Chem 50:4437-4444

175. Davalos A, Gomez-Cordoves C, Bartolome B 2004 Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. J Agric Food Chem 52:48-54

176. Levine CR, Davis JM 2001 Resuscitation with 100% oxygen: should we change our ways? Pediatr Res 50:432

177. Lefkowitz W 2002 Oxygen and resuscitation: beyond the myth. Pediatrics 109:517-519

178. Saugstad O 2003 Oxygen Toxicity at Birth: The Pieces Are Put Together. Pediatr Res 54:789

179. Tan A, Schulze A, O`Donnell C, Davis P 2005 Air versus oxygen for resuscitation of infants at birth. Cochrane Database Syst RevCD002273.pub 3

Errata

Paper III

Results, p 462, second column, Total MMP activity, fourth line should read; “Left ventricle total MMP-activity increased in group A1/B1 (14930·0 R.F.U. ± 922, $P = 0{\cdot}003$) and group A2/B2 (13634·5 R.F.U. ± 1240, $P = 0{\cdot}027$) compared to the control piglets (9606·5 R.F.U. ± 1453)”.