Resuscitation of the newborn with 100% O₂ – detrimental effects on the brain, lungs and heart

An experimental study in piglets

Berit Holthe Munkeby

Department of Pediatric Research and
Institute for Surgical Research
Faculty Division Rikshospitalet
University of Oslo, Norway
2005
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………………………………………………………………………..3

Acknowledgements……………………………………………………………………5

Publications included in the thesis………………………………………………..7

Abbreviations……………………………………………………………………………9

Background…………………………………………………………………………….11

Introduction……………………………………………………………………………..12

Birth asphyxia………………………………………………………………………12

   Epidemiology…………………………………………………………………………12

   Definition……………………………………………………………………………12

   Hypoxic-ischemic encephalopathy………………………………………………13

   Mechanisms………………………………………………………………………..14

Chronic lung disease …………………………………………………………………16

   Definition and epidemiology……………………………………………………16

   Mechanisms………………………………………………………………………..17

   Treatments…………………………………………………………………………18

Cardiovascular dysfunction in birth asphyxia……………………………………18

Apgar score…………………………………………………………………………..20

Oxygen in neonatal medicine…………………………………………………….21

Is it harmful to resuscitate neonates with pure oxygen?………………………22

Neonatal resuscitation………………………………………………………………22

Perinatal hypoxic-ischemic tissue damage………………………………………24

   Oxidative stress………………………………………………………………….24

   Matrix metalloproteinases ……………………………………………………25
Acknowledgements

The present work was carried out at the Department of Pediatric Research and Institute for Surgical Research during the years 2000-2005. For the first two years I was a scholarship holder at the Department of Anaesthesiology, Aker University Hospital, Faculty Division Aker University Hospital, and for the final period at the Faculty Division Rikshospitalet, University of Oslo.

I would like to express my deepest gratitude to my principal supervisor, Professor Ola Didrik Saugstad, for introducing me to the scientific world of paediatric research and for giving me the opportunity to work within the field of resuscitation. I would like to thank him for his ideas, encouragement and never-ending enthusiasm, and for introducing me to the international paediatric research community. I also want to thank my co-supervisor during the first two years of the project, Professor Jan Henrik Rosland, Department of Anaesthesiology, Aker University Hospital, for his advice, constant encouragement and help in completing this work. Special thanks go to co-author Bente Halvorsen, MSc, PhD, for her knowledge, support, criticism, constructive suggestions and helpful comments throughout this work. Many thanks also to the co-authors Anne Beate Solås, MD, PhD, and J. Frederik Frøen, MD, PhD, for introducing me to the piglet model. I also wish to thank Professor Ansgar Aasen, head of the Institute for Surgical Research and his staff, Vivi Bull Stubberud, Sera T. Sebastian and Aurora M. Pamplona who have provided me with excellent working facilities and invaluable help with all kinds of practical details during the animal experiments. My thanks also go to the engineers Grethe Dyrhaug, Julie K. Lindstad, Vincent Maure, Hilde Nilsen, Tove Norèn, Solveig Pettersen and Tarjei Tjønn for excellent technical assistance. Chief veterinarian Dag
Sørensen and engineer Randi Væråmoen at the Department of Comparative Medicine have always been helpful with their assistance, and delivery of piglets. I also want to thank my co-workers: Wenche B. Børke, MD; Kristin Bjørnland, MD, PhD; Liv I.B. Sikkeland, MSc; Grethe I.A.Borge, MSc, PhD; Santiago Rivera, PhD; Michel Khrestchatisky, PhD; Jon Lømo, MD, PhD; Kristin Lyng, MD; Eldrid H.Winther-Larsen, MSc; Professor Hans-Jørgen Smith and Atle Bjørnerud, PhD. Without their participation and effort, this work would not have been feasible. I am grateful to Marianne Wright, PhD for her support, criticism, constructive suggestions, and helpful comments throughout the process. I would also like to thank chief administrative officer Elisabeth Mathiassen and research fellows and friends at the Institute for their support and encouragement during my projects. Professor Thore Egeland, Geir Aamodt, PhD, and Kathrine F. Frøsli, MSc, have generously provided guidance on statistics.

I want to thank my family Hanne, Hans, Rannveig, Gaute, Tove, Eva, Gunnar and Petter for all their valuable support. And finally, my sincere thanks go to my husband Einar, for love, support and encouragement during the experiments, and for help with the manuscripts. Without him I would never have started nor completed this.

My research work has been supported financially by the University of Oslo. It was also supported by the Norwegian SIDS Society, AGA AB Medical Research Fund, the Laerdal Foundation for Acute Medicine, the Norwegian Air Ambulance and the Norwegian Society of Anaesthesiology.
Publications included in the thesis

The papers are referred to by roman numerals in the text:

I  Munkeby, B.H.; Børke, W.B.; Bjørnland, K.; Sikkeland, L.I.B.; Borge, G.I.A.; Halvorsen, B. and Saugstad, O.D.

II Munkeby, B.H.; Børke, W.B.; Bjørnland, K.; Sikkeland, L.I.B.; Borge, G.I.A; Lømo, J; Rivera, S; Khrestchatisky, M.; Halvorsen, B. and Saugstad, O.D
Resuscitation of hypoxic piglets with 100% oxygen increases pulmonary metalloproteinases and IL-8. In press in Pediatric Research, 2005.

III Børke, W.B.; Munkeby, B.H.; Halvorsen, B.; Bjørnland, K; Tunheim, S.H.; Borge, G.I.A.; Thaulow, E.; Saugstad, O.D.
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

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

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
CMRO₂ = 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
PaO₂ = arterial O₂ tension
PaCO₂ = arterial carbon dioxide tension
pCO₂ = pressure of CO₂
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 $\text{Na}^+/\text{K}^+$-ATPase activity. Influx of $\text{Na}^+$ and $\text{Ca}^{2+}$ 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 pO2 of 2.6-3.3 mmHg to an extrauterine environment with a pO2 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, intraventricular 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 O₂ 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 extracellular 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-inflammatoty 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 bronchoalveolar 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 edema 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 PaCO₂ - 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) ⅔, 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 \( \text{O}_2 \) 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.
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 50 μg/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 N₂O (30%) and O₂ (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 (CMRO2) (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/N₂O inhalation and bolus injections with Fentanyl iv were used. The inhalation agent isoflurane as a sole agent or in combination with nitrous oxide (N₂O) has been demonstrated to provide the least cardio depressant effects while providing stable anaesthesia. Pigs are relatively resistant to the analgesic effects of N₂O, 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.

![Principles of microdialysis](image)

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
collection 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).
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% O₂ compared to 21% O₂ (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% O₂ (p < 0.05), and only in the blood vessels after 21% O₂. Compared to baseline, ORAC values were considerably lowered in the resuscitated piglets (p < 0.05) and significantly reduced in the 100% O₂ versus 21% O₂ group (p < 0.05). In BAL fluid, both pro-MMP-9 and pro-MMP-2 increased two-fold in the 100% O₂ group compared to 21% O₂ (p < 0.05). Moreover, IL-8 concentration increased significantly in piglets resuscitated with 100% O₂ compared to 21% O₂ (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% O₂ than with ambient air (p = 0.012). The ORAC value was considerably decreased in piglets resuscitated with 100% O₂ compared with baseline (p < 0.001). In piglets with elevated PaCO₂, 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 PaCO₂ than in piglets with elevated PaCO₂ (p = 0.013). In hypoxaemia-reoxygenation injury the MMP-2 level was highly increased and was most elevated in the piglets resuscitated with 100% O₂. Antioxidant capacity was considerably decreased. Assessed by total the MMP-activity, elevated PaCO₂ during resuscitation might protect the left ventricle, and probably increase the right ventricle injury of the myocardium.
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% O₂ causes more upregulation of early markers of tissue injury than resuscitation with 21% O₂. 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% O₂ 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% O₂ compared with 21% O₂ 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% O₂ compared with 21% O₂. 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 PaCO₂ levels (Paper I and II).

IV  Elevated PaCO₂ 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 PaCO₂ 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


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


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


   Resuscitation with 100% O2 does not protect the myocardium in hypoxic

    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

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


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


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

    Dis Child 50:696-702


    asphyxia. Arch Dis Child 52:620-626

    resuscitation of apparently stillborn infants: survival and long-term outcome. J
    Pediatr 118:778-782


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


76. Volpe JJ 1997 Brain injury in the premature infant--from pathogenesis to prevention. Brain Dev 519-534


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


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


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


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

metalloproteinase inhibition prevents oxidative stress-associated blood-brain
barrier disruption after transient focal cerebral ischemia. J Cereb Blood Flow
Metab 21:1393-1400

Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart.
Circulation 101:1833-1839

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


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


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


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


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


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


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


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


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, \( P = 0·003 \)) and group A2/B2 (13634·5 R.F.U. ± 1240, \( P = 0·027 \)) compared to the control piglets (9606·5 R.F.U. ± 1453)”. 