Less Stress

Oxidative stress and glutathione kinetics in preterm infants

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Oxidatieve stress en glutathion metabolisme in premature pasgeborenen

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General introduction

PREMATURITY

Preterm birth, defined as birth before 37 weeks of gestation, is one of the major causes of mortality and long-term morbidity in perinatal medicine[1] [2]. Preterm infants can be classified according to gestational age (GA); extremely premature (GA < 28 weeks), severely premature (GA 28-31 weeks), moderately premature (GA 32-33 weeks) and near term (GA 34-36 weeks). In Europe, the incidence of preterm birth is reported to be 5-9% of all deliveries, of which 20% are born before 28 weeks of gestation[2-4]. The number of preterm births has been rising over the past decades, which is amongst others due to an increase in indicated preterm deliveries, e.g. earlier intervention in preeclampsia, and increased number of assisted pregnancies.

A main concern is that prematurity accounts for 75% of perinatal mortality and more than half of the long-term morbidity[1-3]. Before the widespread use of assisted ventilation in the 1970s, there were few survivors amongst infants born extremely premature and even the more mature infants died from respiratory distress caused by absence of pulmonary surfactant therapy[5]. With the use of antenatal corticosteroids, improved methods of assisted ventilation and the introduction of surfactant administration, survival rates for preterm infants have improved remarkably. Improved survival, however, has not been accompanied by a corresponding decrease in major neonatal morbidities. The National Institute of Child Health & Human Development (NICHD) Neonatal Research Network reported a decreased mortality of preterm infants, but an increase in major morbidities among infants with a birth weight of 501-750 gram[6].

The question has been raised whether active perinatal and neonatal care might indeed result in an increasing number of survivors who are at higher risk of significant neonatal morbidities. The authors of the EXPRESS study, a Swedish cohort study, stated, "As the ultimate goal of perinatal and neonatal care is intact survival free from neurodevelopmental impairment, the persisting high morbidity rates are of great concern"[7]. Therefore, research in perinatal medicine should be directed to improving the long-term outcome after preterm birth by reducing the occurrence and severity of neonatal morbidities. In the early 1980's, the term 'oxygen radical disease in neonatology' was introduced, relating several serious neonatal diseases through a common pathogenesis, namely oxidative stress[8] [9]. Although the major neonatal diseases have a complex, multi-factorial and poorly understood pathogenesis, oxidative stress seems to play a crucial role[9-15].

OXIDATIVE STRESS

Oxidative stress is the resultant of an imbalance between oxidants and antioxidants. An oxidant is a substance that removes electrons from another substance in a redox reaction, thereby reducing itself and oxidizing the other substance. Reactive oxygen species (ROS) are oxidants

produced during, amongst others, normal oxygen metabolism and include the superoxide anion (O_2^-), the hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). ROS are highly reactive molecules which are able to cause oxidative damage to proteins, lipids and DNA resulting in altered enzyme activities, damage of cellular and mitochondrial membrane, altered signal transduction, and even apoptosis. Besides being toxic, ROS also have crucial beneficial actions, e.g. regulating vascular tone, as part of physiological response to kill pathogens, inter- and intracellular signaling, activating protein cascades, and control gene expression[14-16]. Thus, ROS are essential but are dangerous if in excess.

To counteract the detrimental actions of ROS, the body has numerous antioxidant defense mechanisms (antioxidants), in the form of enzymes, vitamins and other agents (**Table 1**). An antioxidant is defined as "any substance that delays, prevents or removes oxidative damage", thereby counteracting the deleterious effects of oxidants.

Table 1: Antioxidants.

Class	Antioxidant
Enzymes	Superoxide dismutase
	Catalase
	Glutathione peroxidase
	Glutathione reductase
Vitamins	Vitamin E
	Vitamin A
	Vitamin C
	Coenzyme Q
	β-carotene
Reducing agents	Glutathione
	Cysteine
	Thioredoxin
Binding agents	Albumin
	Ceruloplasmin
	Lactoferrin
	Transferrin
Constituent of enzymes	Coper, zinc, selenium
Other	Uric acid
	Bilirubin
	Erythropoietin

GLUTATHIONE

Glutathione (GSH) is one of the most important non-enzymatic intracellular antioxidants. Studies on human hereditary GSH depletion and data on experimental depletion in animal models, demonstrate its importance for survival[17 18]. Dietary GSH is only minimally released in the bloodstream and thus the body is dependent on de novo synthesis of GSH[17 19]. GSH is a tri-peptide synthesized from the amino acids glutamate, cysteine and glycine (**Figure 1**). Although synthesized in all tissues, GSH is mainly synthesized in liver en erythrocytes.

The primary function of GSH is maintaining redox balance[20-24]. This is achieved directly by oxidation of GSH to its dimeric form (GSSG) by GSH peroxidase (GPx), for which the cysteine residue with its reducing sulphydryl group is responsible. GPx reduces hydrogen peroxide by transferring the energy from the reactive peroxides to the sulfur-containing GSH. GSSG can be reconverted to GSH by the enzyme glutathione reductase (GR), using NADPH as source of electrons. The NADPH required in this reaction as a reducing equivalent is mainly provided by the oxidative pentose phosphate pathway. In addition to oxidation to its dimeric form, GSH is capable to directly bind toxic metals and other toxic compounds to its sulphydryl group.

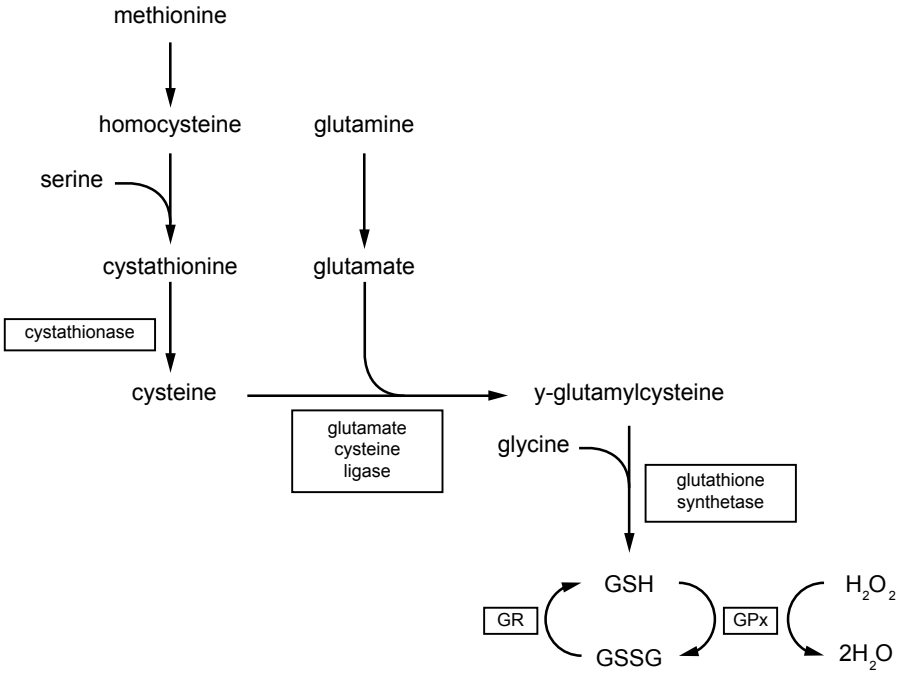

Figure 1: Synthesis of glutathione.
GR = glutathione reductase, GPx = glutathione peroxidase, GSH = reduced glutathione, GSSG = oxidized glutathione

Besides owning intrinsic antioxidant properties, GSH is also an essential cofactor for enzymes such as GSH-S-transferases (GST).

Other than its antioxidant properties, GSH exerts many other functions. By affecting cellular redox status, GSH influences the regulation of signal transduction pathways and gene transcription. The redox status is also important for modulation of several enzymes. GSH maintains the reduced state of the sulfphydryl groups of many proteins, which is required for their normal function. Furthermore, GSH functions as storage and transport of cysteine. In this manner, GSH prevents auto-oxidation of circulating cysteine (thereby forming the highly reactive hydroxyl radical[17]), serves as a cysteine reserve during food deprivation and is source of cysteine for lymphocytes. GSH also plays an important role in the response of the immune system to infections, such as in the synthesis of leukotrienes and in lymphocyte proliferation.

OXIDATIVE STRESS IN NEWBORNS

Birth is accompanied by an abrupt transition from a hypoxic environment in utero (pO_2 20-25 mmHg) to a relative hyperoxic environment ex utero (pO_2 100 mmHg). This sharp increase in oxygen pressure is believed to generate a burst of ROS[25 26] and thus results in physiologic oxidative stress immediately after birth in newborn infants[27]. Besides this hyperoxic challenge, the increase in metabolic rate after birth adds to this physiological oxidative stress[28].

In term infants, antioxidant defenses are present at birth to counteract the hyperoxic challenge at birth. The antioxidant enzymes (SOD and GPx) mature during the last trimester of pregnancy[9 16 28-32]. Also, it has been suggested that there is an increased transfer across the placenta of non-enzymatic antioxidants during the last days of pregnancy[28 30]. Furthermore, term infants have the capability to increase their antioxidant enzymes in the lung in reaction to the sudden O_2 exposure at birth and are also capable of inducing greater levels of antioxidants under conditions of oxidative stress[28 31-34].

Oxidative stress in preterm infants

Preterm infants are highly susceptible to oxidative stress, because they have both increased ROS formation as well as compromised antioxidant defenses. Besides the physiological oxidative challenge at birth, premature infants often require additional oxygen during resuscitation in order to achieve an appropriate rise in oxygen saturation (SpO_2) levels[35]. Oxygen supplementation during resuscitation after birth is associated with increased oxidative stress and this seems to be a dose-dependent association[36 37]. Furthermore, preterm birth is often associated with fetal or maternal morbidities, such as preeclampsia or chorioamnionitis, which are linked to increased oxidative stress[38 39]. Also in the neonatal period, preterm infants are at risk for increased ROS formation. Oxygen dependency due to immature lungs, infections, intravenous lipids and increased free iron due to multiple blood transfusions are thought to contribute to

increased oxygen radical formation[12 40-44]. In the first days and weeks after birth, levels of oxidative stress are increased in preterm infants[45 46].

While ROS formation is increased, the antioxidant defenses are not fully matured or present at birth when an infant is born premature[22 30 45 47 48]. After birth, there is also an increased antioxidant consumption, thereby reducing antioxidant capacity of preterm infants further[49]. It has been shown that GSH concentration decreases rapidly during the first days of life in preterm infants[22 50 51]. This is most likely caused by the increased consumption of GSH due to oxidative stress, but it has also been suggested that GSH synthesis might be hampered by immaturity of the enzymatic apparatus or by lack of substrate.

The nutritional state of the newborn modulates the antioxidant defenses[52 53]. Breast milk is a great source of antioxidant[54 55]. However, preterm infants do not receive high amounts of breast milk in the first days of life[56]. Furthermore, breast milk of mothers after term delivery has higher antioxidant power than that of mothers after preterm delivery[55]. Due to immaturity of the gastrointestinal tract, preterm infants are initially dependent on parenteral nutrition. Previously, Te Braake et al (2008) demonstrated that early amino acid administration resulted in increased availability of GSH[57]. However, this did not result in a reduction of oxidative stress markers. Besides amino acid administration, preterm infants require parenteral lipids for energy and essential fatty acids. However, lipids are prone to lipid peroxidation and are associated with increased oxidative stress[44 58 59].

OXYGEN RADICAL DISEASES OF NEONATOLOGY

Despite the development of neonatal care in the last decades, several severe neonatal diseases continue to pose a major problem, leading to mortality and morbidity after preterm birth. In 1988, dr. Ola Saugstad hypothesized that many of these neonatal diseases share a common pathogenesis through oxidative stress[8 9]. Instead of being different diseases, these are different entities of a common pathway and the clinical manifestations of the disease differ according to the organ most affected[8 9]. He named these diseases the 'oxygen radical diseases in neonatology', which encompasses bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), necrotizing enterocolitis (NEC) and periventricular leukomalacia (PVL). All of these separate diseases share a complex, multi-factorial and poorly understood pathogenesis, in which ROS seem to play a crucial role[9-15].

Bronchopulmonary dysplasia

Bronchopulmonary dysplasia (BPD), also referred to as chronic lung disease of prematurity, is the most common chronic lung disease of childhood. BPD is defined as the need for oxygen supplementation for at least 28 days and its severity is categorized by the need for oxygen and/or positive pressure at 36 weeks postmenstrual age[60]. The reported incidence of BPD

varies widely and is dependent on the gestational age and birth weight of the cohort. Among infants with a birth weight <1500 gram, the incidence varies between 5 and 40%[5 9]. Nowadays, increased numbers of very immature children with an earlier pulmonary developmental stage survive. Whereas the first generations of BPD patients were born with lungs in the early alveolar stage and BPD was characterized by fibrosis and atelectasis ('old BPD'), the very immature infants of today's generation are born with lungs in the saccular stage and lung damage is accompanied by arrest of alveolar growth and larger alveoli with lowered capillary density ('new BPD')[60].

The precise pathogenesis of BPD remains unclear, but is clearly multifactorial. Lung immaturity, as seen in (extreme) prematurity, is the most important predisposing factor. However, oxygen administration and ventilation also play an important role[9]. Immediately after the first description of BPD by Northway et al. in 1967, the authors linked the condition to oxygen toxicity[61]. Since then, many studies have implicated ROS in the pathogenesis of BPD[15 62-64].

Retinopathy of prematurity

Retinopathy of prematurity (ROP) is a vasoproliferative disorder of the retina of preterm infants in which the vascularisation of the immature retina is disturbed during the first weeks of extrauterine life. In its most severe form, ROP can lead to retinal blindness and it is a main cause of blindness in the United States[65]. ROP was the first disease associated with oxygen, after an epidemic of blindness was witnessed in the 1940's after introduction of supplemental oxygen therapy in neonatology[66]. Since then, the incidence of blindness due to ROP remarkably reduced as a result of restricted oxygen use and cryotherapy to treat ROP[67 68].

Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in preterm infants. The incidence varies highly among countries and even among different centers within the same country. Reported incidences, therefore, range from 1-10% among preterm infants with mortality rates ranging from 10% to 30%[6 69 70]. The pathogenesis of NEC appears to be multifactorial where prematurity and formula feeding are the most important risk factors. NEC seems to occur by the coincidence of intestinal ischemia, colonization by pathogenic bacteria and excess protein substrate in the intestinal lumen[71]. Increased ROS production also has been implicated in the occurrence of NEC, probably by mediating the microvascular and mucosal permeability[72-74].

Periventricular leukomalacia

Periventricular leukomalacia (PVL) results from degeneration of white matter adjacent to the cerebral ventricles. The pathogenesis of PVL includes an incomplete development of the vascular supply to the cerebral white matter and a maturation-dependent impairment in auto-

regulation of the cerebral blood flow[75 76]. A third major factor in the pathogenesis of PVL seems to be oxidative stress.

The brain is particularly susceptible to oxidative damage, since neuronal membranes are rich in polyunsaturated fatty acids. Also, differentiating oligodendrocytes are particularly vulnerable to oxidative damage since these are rich in iron which is needed for differentiation[77]. There seems to be a maturation-dependent vulnerability of the oligodendroglial precursor cell that represents the major cellular target in PVL, which could explain why only white matter is selectively damaged in the preterm brain[78].

MEASUREMENT OF OXIDATIVE STRESS

Besides the occurrence of the so-called 'oxygen radical diseases of neonatology', oxidative stress can be determined through measurement of ROS, measurement of products of oxidative damage and measurement of the antioxidant defense.

Oxidative stress markers

Most ROS are very unstable molecules and will readily react with other molecules in order to stabilize themselves. They can be measured with techniques like spin trapping. This technique involves the addition of a radical to a nitrone spin trap resulting in the formation of a spin adduct, a nitroxide-based persistent radical, that can be detected using electron paramagnetic resonance (EPR) spectroscopy. We have chosen to measure end-products of oxidative damage as a marker of oxidative stress. ROS cause oxidative damage to proteins, DNA and lipids and a wide variety of oxidative stress markers are available.

In this thesis, we mainly determined oxidative stress markers in urine. The studies in this thesis are focused on preterm infants, which limits the use of large blood samples for research purposes. Urine, however, is freely available and poses no additional risk for the preterm infant. It can be collected by placing gauzes in an infant's diaper during routine care. We used well-known and reliable biomarkers of oxidative stress, nitrosative stress and inflammation due to free radicals. In the nutritional intervention studies we also determined urinary F_2-isoprostanes as a marker of lipid peroxidation.

Besides urinary oxidative stress markers, we also determined non protein bound iron (NPBI) in plasma, which only requires a small blood sample. Normally, iron is mostly bound to ferritin or other proteins. However, increased oxidative stress causes iron to be released from storage. In turn, free iron increases oxidative stress by promoting the release of hydroxyl radicals via the Fenton reaction[79]. NPBI is, therefore, a general marker of oxidative stress[80].

Glutathione

As mentioned previously, GSH is the main intracellular antioxidant and its measurement provides insight in the antioxidant response to an oxidative insult. In this thesis, GSH concentration was measured in erythrocytes of preterm infants. Besides being readily accessible as opposed to other tissues, erythrocytes are also suggested to function as antioxidant defense by being a physiological source of GSH and by taking up ROS[22 81]. Giustarini et al. provided strong evidence for a role of erythrocytes as GSH donor for other tissues[81]. Furthermore, membranes of erythrocytes are permeable to superoxide and hydrogen peroxide, thereby protecting other tissues against oxidative damage.

Determination of GSH concentrations provides only a static measurement and thus quantification of synthesis rates, using a stable isotope study, is far more interesting to study its metabolism under pathological conditions, such as oxidative stress, or in response to interventions. As mentioned previously, GSH can be oxidized to its dimeric form GSSG thereby reducing hydrogen peroxide. GSH is mostly kept in reduced form by glutathione reductase (GR), with concentrations of GSSG being 1/1000 of the total glutathione concentration[82]. Measurement of the ratio between GSH and GSSG has been considered an index of the whole-organism oxidative status[82 83].

OXYGEN AS A DRUG IN NEONATOLOGY

Although the ancient Greeks identified air as one of the four elemental components of creation, oxygen was not described until the late 18th century. Around the same time, the English chemist Joseph Priestly, the Swedish pharmacist Carl Wilhelm Scheele and the French chemist Antoine Lavoisier all studied and described their findings on the gas later known to be oxygen. Within a few years after its discovery, oxygen was used in medicine. The first reported case in which oxygen was actually used as a remedy was a case of tuberculosis in a young woman who benefited by daily inhalations of oxygen (published in the Gazette de Sante).

In 1781, Francois Chaussier constructed an apparatus for resuscitation of the newborn infant with a bag and mask that allowed artificial ventilation. He proposed the use of oxygen in the revival of 'near-dead' infants. It took another 100 years before oxygen was used to treat preterm infants. In 1889, Dr. Tarnier decided to administer oxygen in the incubator of a preterm infant for two hours a day until it was strong enough to leave the incubator (published in Journal de Medecine de Paris, 1891). It was not until 1934 that oxygen therapy became a standard treatment for preterm infants when Dr. Julius Hess modified incubators, before then only used to warm infants, with free flow oxygen. In 1938, Dr. Chapple further developed the incubator, which was reported to maintain a constant oxygen concentration of 46%[84].

Oxygen toxicity

It was Priestley who, in 1775, first raised concern: "Though pure dephlogisticated air might be very useful as a medicine, it might not be so proper for us in the usual healthy state of the body for, as a candle burns out much faster in dephlogisticated air than in common air, so we might, as may be said, live out too fast and the animal powers be too soon exhausted in this pure kind of air. A moralist, at least, may say, that the air which nature has provided for us is as good as we deserve." (Priestley J. *Experiments and observations on different kinds of air*. London: Johnson and St Paul's Churchyard; 1775).

Pediatricians noticed in the late 1940's that, after the use of oxygen was introduced for the treatment of preterm infants, many infants turned blind and they discovered that this was caused by retrolental fibroplasias (later renamed to ROP). In 1951, Dr Cambell was the first to describe oxygen as a key accomplice in ROP[66]. Around the same time, it was postulated that oxygen is toxic due to its tendency to generate oxygen radicals[85]. Attempts were made to control oxygen use in the management of preterm infants, but limited use of oxygen initially caused increased mortality rates and cerebral palsy. Finally, it was recognized that both hypoxia and hyperoxia, as monitored by transcutaneous measurement of the PaO_2 and nowadays by pulse oximetry, should be avoided[15 86-88]. However, the optimal SpO_2 range for preterm infants is still subject of debate, which is underscored by the fact that Data Monitoring Committees closed inclusion in a trial comparing high and low target ranges due to large differences in mortality[89 90].

AIMS & OUTLINE OF THIS THESIS

The general aim of this thesis was to study oxidative stress in preterm infants and to explore possible options to reduce the impact of oxidative stress in neonatal care.

The first aim of this thesis was to perfect the technique of measuring GSH kinetics. Since research in preterm infants is limited by small blood volumes, it is important to reliably measure GSH enrichments and concentrations in small samples. **Chapter 2** describes the simultaneous analysis of both GSH and its precursor glycine, thereby reducing the blood volume needed to measure GSH kinetics.

The second aim of this thesis was to reduce oxidative stress at birth. Since both hypoxia and hyperoxia during the critical timeframe after birth can have long lasting effects, it is important to determine the optimal oxygen concentration for the resuscitation at birth. In **Chapters 3 - 5**, the study protocol and main results of a randomized, double-blinded study comparing safety and efficacy of 30% versus 65% oxygen for the resuscitation of preterm infants at birth are presented. After the initial fraction of inspired oxygen, oxygen administration is titrated

based on the oxygen saturation measured by pulse oximetry. **Chapter 6** describes the current performance of saturation management during resuscitation in daily practice.

The third aim of this thesis was to study the modifying effect of nutrition on oxidative stress. Nutrition might have a beneficial effect on the antioxidant capacity of preterm infants, thereby improving outcome of preterm infants. However, nutrition, and especially lipids, has been implicated in increasing oxidative stress. It is, therefore, important to optimize nutrition without adding to the oxidative burden of preterm infants. **Chapter 7** describes an observational study on the effects of early AA administration of preterm infants. **Chapter 8** describes a randomized clinical trial on the effect of early intravenous nutrition on oxidative stress in preterm infants. In **Chapter 9**, we studied the effect of administration of two different lipid emulsions on oxidative stress in preterm infants.

Finally, we investigated other modulators of oxidative stress in preterm infants. In **Chapter 10**, we studied the effects of nosocomial sepsis on antioxidant availability in preterm infants. **Chapter 11** describes the effect of gender, gestational age and birth weight on GSH synthesis and GSH recycling. To reduce the oxidative insult as a consequence of oxygen administration in the weeks following birth, we evaluated the hazards in the process of supplemental oxygen therapy (**Chapter 12**).

The main conclusions of this thesis, together with recommendations for future research and a general discussion on oxidative stress in preterm infants are presented in **Chapter 13**.

REFERENCES

1. Lawn JE, Wilczynska-Ketende K, Cousens SN. Estimating the causes of 4 million neonatal deaths in the year 2000. *International journal of epidemiology* 2006;35(3):706-18.
2. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet* 2008;371(9606):75-84.
3. Muglia LJ, Katz M. The enigma of spontaneous preterm birth. *The New England journal of medicine* 2010;362(6):529-35.
4. Stichting Perinatale Registratie Nederland.
5. Saigal S, Doyle LW. An overview of mortality and sequelae of preterm birth from infancy to adulthood. *Lancet* 2008;371(9608):261-9.
6. Stoll BJ, Hansen NI, Bell EF, Shankaran S, Laptook AR, Walsh MC, et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics* 2010;126(3):443-56.
7. EXPRESS-group. Incidence of and Risk Factors for Neonatal Morbidity after Active Perinatal Care: Extremely Preterm Infants Study in Sweden (EXPRESS). *Acta Paediatr* 2010;99(7):978-92.
8. Saugstad OD. Hypoxanthine as an indicator of hypoxia: its role in health and disease through free radical production. *Pediatr Res* 1988;23(2):143-50.
9. Saugstad OD. The oxygen radical disease in neonatology. *Indian J Pediatr* 1989;56(5):585-93.
10. Rogers S, Witz G, Anwar M, Hiatt M, Hegyi T. Antioxidant capacity and oxygen radical diseases in the preterm newborn. *Arch Pediatr Adolesc Med* 2000;154(6):544-8.
11. Saugstad OD, Hallman M, Abraham JL, Epstein B, Cochrane C, Gluck L. Hypoxanthine and oxygen induced lung injury: a possible basic mechanism of tissue damage? *Pediatr Res* 1984;18(6):501-4.
12. Dennery PA. Role of redox in fetal development and neonatal diseases. *Antioxid Redox Signal* 2004; 6(1):147-53.
13. Sola A, Rogido MR, Deulofeut R. Oxygen as a neonatal health hazard: call for detente in clinical practice. *Acta Paediatr* 2007;96(6):801-12.
14. Saugstad OD. Update on oxygen radical disease in neonatology. *Curr Opin Obstet Gynecol* 2001;13(2): 147-53.
15. Saugstad OD. Oxidative stress in the newborn--a 30-year perspective. *Biol Neonate* 2005;88(3):228-36.
16. Saugstad OD. Oxygen and oxidative stress in bronchopulmonary dysplasia. *J Perinat Med* 2010;38(6): 571-7.
17. Kidd PM. Glutathione: Systemic protectant against oxidative and free radical damage. *Alt Med Rev* 1997;2(3):155-76.
18. Ristoff E, Larsson A. Oxidative stress in inborn errors of metabolism: lessons from glutathione deficiency. *Journal of inherited metabolic disease* 2002;25(3):223-6.
19. Witschi A, Reddy S, Stofer B, Lauterburg BH. The systemic availability of oral glutathione. *European journal of clinical pharmacology* 1992;43(6):667-9.
20. Malmezat T, Breuille D, Capitan P, Mirand PP, Obled C. Glutathione turnover is increased during the acute phase of sepsis in rats. *The Journal of nutrition* 2000;130(5):1239-46.
21. Lyons J, Rauh-Pfeiffer A, Ming-Yu Y, Lu XM, Zurakowski D, Curley M, et al. Cysteine metabolism and whole blood glutathione synthesis in septic pediatric patients. *Crit Care Med* 2001;29(4):870-7.
22. Ahola T, Levonen AL, Fellman V, Lapatto R. Thiol metabolism in preterm infants during the first week of life. *Scand J Clin Lab Invest* 2004;64(7):649-58.
23. Hansson L, Seidegard J, Johansson L, Jeppsson B. Influence of glutathione metabolising enzymes in rats with gram-negative sepsis. *Eur J Surg* 2000;166(9):728-33.
24. Biolo G, Antonione R, De Cicco M. Glutathione metabolism in sepsis. *Crit Care Med* 2007;35(9 Suppl): S591-5.
25. Vento M, Asensi M, Sastre J, Lloret A, Garcia-Sala F, Minana JB, et al. Hyperoxemia caused by resuscitation with pure oxygen may alter intracellular redox status by increasing oxidized glutathione in asphyxiated newly born infants. *Seminars in perinatology* 2002;26(6):406-10.

26. Comporti M, Signorini C, Leoncini S, Buonocore G, Rossi V, Ciccoli L. Plasma F2-isoprostanes are elevated in newborns and inversely correlated to gestational age. *Free radical biology & medicine* 2004;37(5):724-32.
27. Pallardo FV, Sastre J, Asensi M, Rodrigo F, Estrela JM, Vina J. Physiological changes in glutathione metabolism in foetal and newborn rat liver. *Biochem J* 1991;274 (Pt 3):891-3.
28. Friel JK, Friesen RW, Harding SV, Roberts LJ. Evidence of oxidative stress in full-term healthy infants. *Pediatr Res* 2004;56(6):878-82.
29. Vina J, Vento M, Garcia-Sala F, Puertes IR, Gasco E, Sastre J, et al. L-cysteine and glutathione metabolism are impaired in premature infants due to cystathionase deficiency. *Am J Clin Nutr* 1995;61(5): 1067-9.
30. Davis JM, Auten RL. Maturation of the antioxidant system and the effects on preterm birth. *Semin Fetal Neonatal Med* 2010.
31. Munoz-Hoyos A, Bonillo-Perales A, Avila-Villegas R, Gonzalez-Ripoll M, Uberos J, Florido-Navio J, et al. Melatonin Levels during the First Week of Life and Their Relation with the Antioxidant Response in the Perinatal Period. *Neonatology* 2007;92(3):209-16.
32. Frank L, Groseclose EE. Preparation for birth into an O2-rich environment: the antioxidant enzymes in the developing rabbit lung. *Pediatr Res* 1984;18(3):240-4.
33. Asikainen TM, White CW. Pulmonary antioxidant defenses in the preterm newborn with respiratory distress and bronchopulmonary dysplasia in evolution: implications for antioxidant therapy. *Antioxid Redox Signal* 2004;6(1):155-67.
34. Frank L, Sosenko IR. Failure of premature rabbits to increase antioxidant enzymes during hyperoxic exposure: increased susceptibility to pulmonary oxygen toxicity compared with term rabbits. *Pediatr Res* 1991;29(3):292-6.
35. Shoji H, Koletzko B. Oxidative stress and antioxidant protection in the perinatal period. *Curr Opin Clin Nutr Metab Care* 2007;10(3):324-8.
36. Solberg R, Andresen JH, Escrig R, Vento M, Saugstad OD. Resuscitation of hypoxic newborn piglets with oxygen induces a dose-dependent increase in markers of oxidation. *Pediatr Res* 2007;62(5):559-63.
37. Vento M, Moro M, Escrig R, Arruza L, Villar G, Izquierdo I, et al. Preterm Resuscitation With Low Oxygen Causes Less Oxidative Stress, Inflammation, and Chronic Lung Disease. *Pediatrics* 2009;124(3):e439-49.
38. Cheah FC, Pillow JJ, Kramer BW, Polglase GR, Nitsos I, Newnham JP, et al. Airway inflammatory cell responses to intra-amniotic lipopolysaccharide in a sheep model of chorioamnionitis. *Am J Physiol Lung Cell Mol Physiol* 2009;296(3):L384-93.
39. Raijmakers MT, Roes EM, Poston L, Steegers EA, Peters WH. The transient increase of oxidative stress during normal pregnancy is higher and persists after delivery in women with pre-eclampsia. *Eur J Obstet Gynecol Reprod Biol* 2008;138(1):39-44.
40. Moison RM, van Hoof EJ, Clahsen PC, van Zoeren-Grobben D, Berger HM. Influence of plasma preparations and donor red blood cells on the antioxidant capacity of blood from newborn babies: an in vitro study. *Acta Paediatr* 1996;85(2):220-4.
41. Hirano K, Morinobu T, Kim H, Hiroi M, Ban R, Ogawa S, et al. Blood transfusion increases radical promoting non-transferrin bound iron in preterm infants. *Archives of disease in childhood. Fetal and neonatal edition* 2001;84(3):F188-93.
42. Kapoor K, Basu S, Das BK, Bhatia BD. Lipid peroxidation and antioxidants in neonatal septicemia. *J Trop Pediatr* 2006;52(5):372-5.
43. von Dessauer B, Bongain J, Molina V, Quilodran J, Castillo R, Rodrigo R. Oxidative stress as a novel target in pediatric sepsis management. *J Crit Care* 2010.
44. Laborie S, Lavoie JC, Chessex P. Increased urinary peroxides in newborn infants receiving parenteral nutrition exposed to light. *J Pediatr* 2000;136(5):628-32.

45. Buonocore G, Perrone S, Longini M, Vezzosi P, Marzocchi B, Paffetti P, et al. Oxidative stress in preterm neonates at birth and on the seventh day of life. *Pediatr Res* 2002;52(1):46-9.
46. Perrone S, Mussap M, Longini M, Fanos V, Bellieni CV, Proietti F, et al. Oxidative kidney damage in preterm newborns during perinatal period. *Clinical biochemistry* 2007;40(9-10):656-60.
47. Frank L. Antioxidants, nutrition, and bronchopulmonary dysplasia. *Clinics in perinatology* 1992;19(3): 541-62.
48. Georgeson GD, Szony BJ, Streitman K, Varga IS, Kovacs A, Kovacs L, et al. Antioxidant enzyme activities are decreased in preterm infants and in neonates born via caesarean section. *Eur J Obstet Gynecol Reprod Biol* 2002;103(2):136-9.
49. Buhimschi IA, Buhimschi CS, Pupkin M, Weiner CP. Beneficial impact of term labor: nonenzymatic antioxidant reserve in the human fetus. *Am J Obstet Gynecol* 2003;189(1):181-8.
50. Jean-Baptiste D, Rudolph N. Sequential postnatal changes in erythrocyte glutathione and sulfhydryl content: a possible adaptational response to the extrauterine environment. *Biol Neonate* 2003;84(2): 142-6.
51. Jain A, Mehta T, Auld PA, Rodrigues J, Ward RF, Schwartz MK, et al. Glutathione metabolism in newborns: evidence for glutathione deficiency in plasma, bronchoalveolar lavage fluid, and lymphocytes in prematures. *Pediatr Pulmonol* 1995;20(3):160-6.
52. Perrone S, Salvi G, Bellieni CV, Buonocore G. Oxidative stress and nutrition in the preterm newborn. *JPGN* 2007;45:S178-S82.
53. Yeung MY. Influence of early postnatal nutritional management on oxidative stress and antioxidant defence in extreme prematurity. *Acta Paediatr* 2006;95(2):153-63.
54. Friel JK, Martin SM, Langdon M, Herzberg GR, Buettner GR. Milk from mothers of both premature and full-term infants provides better antioxidant protection than does infant formula. *Pediatr Res* 2002; 51(5):612-8.
55. L'Abbe MR, Friel JK. Superoxide dismutase and glutathione peroxidase content of human milk from mothers of premature and full-term infants during the first 3 months of lactation. *Journal of pediatric gastroenterology and nutrition* 2000;31(3):270-4.
56. Corpeleijn WE, Kouwenhoven SM, Paap MC, van Vliet I, Scheerder I, Muizer Y, et al. Intake of Own Mother's Milk during the First Days of Life Is Associated with Decreased Morbidity and Mortality in Very Low Birth Weight Infants during the First 60 Days of Life. *Neonatology* 2012;102(4):276-81.
57. Te Braake FW, Schierbeek H, de Groof K, Vermes A, Longini M, Buonocore G, et al. Glutathione synthesis rates after amino acid administration directly after birth in preterm infants. *Am J Clin Nutr* 2008; 88(2):333-9.
58. Helbock HJ, Motchnik PA, Ames BN. Toxic hydroperoxides in intravenous lipid emulsions used in preterm infants. *Pediatrics* 1993;91(1):83-7.
59. Lavoie PM, Lavoie JC, Watson C, Rouleau T, Chang BA, Chessex P. Inflammatory response in preterm infants is induced early in life by oxygen and modulated by total parenteral nutrition. *Pediatr Res* 2010;68(3):248-51.
60. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 2001;163(7):1723-9.
61. Northway WH, Jr., Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. *The New England journal of medicine* 1967;276(7): 357-68.
62. Gitto E, Pellegrino S, Gitto P, Barberi I, Reiter RJ. Oxidative stress of the newborn in the pre- and postnatal period and the clinical utility of melatonin. *J Pineal Res* 2009;46(2):128-39.
63. Ahola T, Fellman V, Kjellmer I, Raivio KO, Lapatto R. Plasma 8-isoprostane is increased in preterm infants who develop bronchopulmonary dysplasia or periventricular leukomalacia. *Pediatr Res* 2004; 56(1):88-93.
64. Jobe AH, Hillman N, Polglase G, Kramer BW, Kallapur S, Pillow J. Injury and inflammation from resuscitation of the preterm infant. *Neonatology* 2008;94(3):190-6.

65. Steinkuller PG, Du L, Gilbert C, Foster A, Collins ML, Coats DK. Childhood blindness. *Journal of AAPOS : the official publication of the American Association for Pediatric Ophthalmology and Strabismus / American Association for Pediatric Ophthalmology and Strabismus* 1999;3(1):26-32.
66. Campbell K. Intensive oxygen therapy as a possible cause of retrolental fibroplasia; a clinical approach. *The Medical journal of Australia* 1951;2(2):48-50.
67. Chen ML, Guo L, Smith LE, Dammann CE, Dammann O. High or low oxygen saturation and severe retinopathy of prematurity: a meta-analysis. *Pediatrics* 2010;125(6):e1483-92.
68. Lutty GA, Chan-Ling T, Phelps DL, Adamis AP, Berns KI, Chan CK, et al. Proceedings of the Third International Symposium on Retinopathy of Prematurity: an update on ROP from the lab to the nursery (November 2003, Anaheim, California). *Molecular vision* 2006;12:532-80.
69. Kafetzis DA, Skevaki C, Costalos C. Neonatal necrotizing enterocolitis: an overview. *Current opinion in infectious diseases* 2003;16(4):349-55.
70. Lin PW, Stoll BJ. Necrotising enterocolitis. *Lancet* 2006;368(9543):1271-83.
71. Kosloske AM. Pathogenesis and prevention of necrotizing enterocolitis: a hypothesis based on personal observation and a review of the literature. *Pediatrics* 1984;74(6):1086-92.
72. Perrone S, Tataranno ML, Negro S, Cornacchione S, Longini M, Proietti F, et al. May oxidative stress biomarkers in cord blood predict the occurrence of necrotizing enterocolitis in preterm infants? *J Matern Fetal Neonatal Med* 2012;25 Suppl 1:128-31.
73. Granger DN, Rutili G, McCord JM. Superoxide radicals in feline intestinal ischemia. *Gastroenterology* 1981;81(1):22-9.
74. Parks DA, Bulkley GB, Granger DN, Hamilton SR, McCord JM. Ischemic injury in the cat small intestine: role of superoxide radicals. *Gastroenterology* 1982;82(1):9-15.
75. Volpe JJ. Neurobiology of periventricular leukomalacia in the premature infant. *Pediatr Res* 2001; 50(5):553-62.
76. Inder TE, Volpe JJ. Mechanisms of perinatal brain injury. *Seminars in neonatology : SN* 2000;5(1):3-16.
77. Buonocore G, Perrone S, Bracci R. Free radicals and brain damage in the newborn. *Biol Neonate* 2001; 79(3-4):180-6.
78. Back SA, Gan X, Li Y, Rosenberg PA, Volpe JJ. Maturation-dependent vulnerability of oligodendrocytes to oxidative stress-induced death caused by glutathione depletion. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1998;18(16):6241-53.
79. Buonocore G, Perrone S, Longini M, Paffetti P, Vezzosi P, Gatti MG, et al. Non protein bound iron as early predictive marker of neonatal brain damage. *Brain* 2003;126(Pt 5):1224-30.
80. Bracci R, Buonocore G. The antioxidant status of erythrocytes in preterm and term infants. *Seminars in neonatology : SN* 1998;3:191-7.
81. Giustarini D, Milzani A, Dalle-Donne I, Rossi R. Red blood cells as a physiological source of glutathione for extracellular fluids. *Blood Cells Mol Dis* 2007;40(2):174-9.
82. Pastore A, Federici G, Bertini E, Piemonte F. Analysis of glutathione: implication in redox and detoxification. *Clin Chim Acta* 2003;333(1):19-39.
83. Rossi R, Milzani A, Dalle-Donne I, Giustarini D, Lusini L, Colombo R, et al. Blood glutathione disulfide: in vivo factor or in vitro artifact? *Clinical chemistry* 2002;48(5):742-53.
84. Chapple CC. An incubator for infants. *Am J Obstet Gynecol* 1938;35:1062-5.
85. Gerschman R, Gilbert DL, Nye SW, Dwyer P, Fenn WO. Oxygen poisoning and x-irradiation: a mechanism in common. *Science* 1954;119(3097):623-6.
86. Silverman WA. A cautionary tale about supplemental oxygen: the albatross of neonatal medicine. *Pediatrics* 2004;113(2):394-6.
87. Deulofeut R, Critz A, Adams-Chapman I, Sola A. Avoiding hyperoxia in infants < or = 1250 g is associated with improved short- and long-term outcomes. *J Perinatol* 2006;26(11):700-5.
88. Dennery PA. Oxygen administration in the care of neonates: a double-edged sword. *Chin Med J (Engl)* 2010;123(20):2938-42.

89. Stenson B, Brocklehurst P, Tarnow-Mordi W. Increased 36-week survival with high oxygen saturation target in extremely preterm infants. *The New England journal of medicine* 2011;364(17):1680-2.
90. Tarnow-Mordi WO, Darlow B, Doyle L. Target ranges of oxygen saturation in extremely preterm infants. *The New England journal of medicine* 2010;363(13):1285; author reply 85-6.

Chapter 2

Simultaneous analysis of ^{13}C-glutathione as its dimeric form GSSG and its precursor [1-^{13}C] glycine using Liquid Chromatography Isotope Ratio Mass Spectrometry (LC-IRMS)

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ABSTRACT

Introduction

Determination of glutathione kinetics using stable isotopes requires accurate measurement of the tracers and tracees. Previously, the precursor and synthesized product were measured with two separate techniques, liquid chromatography isotope ratio mass spectrometry (LC-IRMS) and gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). In order to reduce sample volume and minimize analytical effort we developed a method to simultaneously determine ^{13}C-glutathione as its dimeric form (GSSG) and its precursor [1-^{13}C] glycine in a small volume of erythrocytes in one single analysis.

Methods

After having transformed ^{13}C-glutathione into its dimeric form GSSG, we determined both the intra-erythrocytic concentrations and the ^{13}C-isotopic enrichment of GSSG and glycine in 150 µL of whole blood using liquid chromatography coupled to LC-IRMS.

Results

The results show that the concentration (range of µmol/mL) was reliably measured using cycloleucine as internal standard, i.e. with a precision better than 0.1 µmol/mL. The ^{13}C-isotopic enrichment of GSSG and Glycine measured in the same run gave reliable values with excellent precision (SD < 0.3 ‰) and accuracy (measured between 0 and 5 APE).

Conclusions

This novel method opens up a variety of kinetic studies with relatively low dose administration of tracers, reducing the total cost of the study design. In addition, only minimal sample volume is required, enabling studies even in very small subjects, such as preterm infants.

INTRODUCTION

Glutathione metabolism has been studied in several different experimental settings and species[1-5]. Glutathione is primarily an intracellular antioxidant, with extremely high cellular concentration (low millimolar range) in mammalian tissue. Quantification of the utilization and synthesis rates provides a dynamic insight into its metabolism under pathological conditions, such as oxidative stress, or in response to interventions. Glutathione is present in two forms: the monomeric, reduced or active form (GSH), and the dimeric, oxidized form (GSSG). GSH is synthesized in a series of two reactions. In the first, rate-limiting step, a glutamate is covalently bound to a cysteine residue. During the second reaction, glycine is added to complete the synthesis[2 6 7]. Glutathione typically has a rapid turnover. The fractional synthesis rate (FSR) reported in healthy adult volunteers varied from 63 to 83%/day in the various studies[5 8 9]. In neonates it was found to be much lower, from 35 to 55%/day[8 10]. Nevertheless, in all cases the total pool is renewed within three days.

FSR determination of GSH in erythrocytes requires suitable methods to measure both low levels of isotopic enrichment in GSH and in its precursor. Moreover, determination in neonates is complicated because only small amounts of blood can be sampled. Studies in neonates would benefit form a method that can deal with small samples. Gas chromatography-mass spectrometry (GC-MS) and stable isotope dilutions techniques have been frequently used in metabolic kinetic studies[11-16] or concentration measurements[17 18] (gold standard method). Several papers have been published over the past four years[19-27], showing the power and robustness of LC-IRMS analyzing amino acids, carbohydrates, fatty acids and volatile fatty acids.

Recently, we have developed a method for measurement of GSSG using liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS)[28]. Still, this method needed to be complemented with glycine measurement on GCMS or GC/C/IRMS. This new method was successfully applied in a kinetic study in preterm infants[10]. In the present study we aimed to develop a new method for simultaneous measurement of ^{13}C-glutathione as its dimeric form (GSSG) and its precursor [1-^{13}C] glycine in erythrocytes, in order to reduce sample preparation and sample volume.

EXPERIMENTAL SECTION

Chemicals and reagents

Glutathione, glycine, cycloleucine and phosphoric acid (85% v/v) were purchased from Sigma (St Louis, USA). Sodium peroxodisulfate (p.a.) was purchased from Fluka (Buchs, Switzerland). Perchloric acid (70% v/v), potassium hydroxide, Na_2HPO_4, H_3PO_4 and sodium hydroxide were purchased from Merck (Darmstadt, Germany). [1-^{13}C] glycine was purchased from Cambridge Isotope Laboratories (Buchem, Apeldoorn, Netherlands). [1,2-^{13}C, ^{15}N] glutathione was a gift

from Cambridge Isotope Laboratory, CIL (Andover, MA, USA). Milli-Q (18.2 MΩ) water was produced with a milli-Q system (Millipore BV, Bedford, MA, USA).

Analytical methods

Experiments were carried out on a Delta XP (Thermo Fisher, Bremen, Germany). The IRMS was operated at an accelerating voltage of 5 kV. The ion source was held at a pressure of 3.0×10^{-6} Torr, and ions generated by electron impact at 70 eV. Three faraday cup detectors simultaneously and continuously monitored the CO_2+• signals for the three major ions at *m/z* 44 ($^{12}CO_2$), *m/z* 45 ($^{13}CO_2$ and $^{12}C^{17}O^{16}O$) and *m/z* 46 ($^{12}C^{18}O^{16}O$). The dynamic range of the instrument is between 0.2 and 50 V. The CO_2 working reference gas quality 5.3 (Linde, Schiedam, Netherlands) was calibrated with known reference gases (Messer Griesheim, Krefeld, Germany) against $\delta\ ^{13}C_{VPDB}$ (Vienna Pee Dee Belemnite, VPDB). In order to increase filament lifetime, the oxygen signal (*m/z* 32) produced by oxidant reagent should not exceed 15 V measured on the first faraday cup (resistor 300 MΩ). A LC-Isolink® interface (Thermo Fisher, Bremen, Germany) was coupled to the Delta XP with slight modifications. The apparatus' conditions were the same as described previously except for some minor modifications[28]. The temperature of the interface reactor was set at 99.9°C. PEEK® tubing was used for connections between the autosampler, the LC column and the interface. In addition, the reagent bottles were degassed with helium during analysis. To avoid crystallisation of reagents the pump heads of the oxidant and acid pumps were rinsed with water several times a day. A filter of 0.2 µm (Vici, Bester BV, Netherlands) was placed between the analytical column and the mixing chamber of the interface to avoid any blockage of particles in the system. Also, a 0.2 µm filter was placed after the reagent pumps to prevent blocking of the oxidation oven by impurities or crystallization deriving from the reagents.

The LC-IRMS interface was coupled to an LC system consisting of two Knauer pumps (Berlin, Germany) and a Midas auto sampler (Spark, Emmen, Netherlands) and controlled by Sparklink software (version 3.10, service pack 2, Spark). The isodat software was used to control the IRMS system.

Calibration and isotopic rearrangements

CO_2 reference gas was introduced at regular 20s intervals at the beginning of each run at a level of 3.0 ± 0.2 V on cup one (resistor 300 MΩ) to calibrate peaks eluting during the run.

The $^{13}C/^{12}C$ abundance ratio was expressed as $\delta\ ^{13}C$ values calibrated against the international standard. The delta notation is defined as $\delta\ ^{13}C_{\ sample} = [(R_s / R_{st}) - 1] \times 1000$, where R_s is the ratio of ^{13}C in the sample and R_{st} is the ratio of the international standard used. The result of this calculation is a relative δ, calibrated against the international standard.

Atom % was calculated as:

$$\text{Atom \%} = \left[\frac{100 \times R \times ((\delta 13c / 1000)+1)}{1+ R \times ((\delta 13c / 1000) -1)} \right]$$

where R is the ratio of (^{13}C/^{12}C) of International Standard of Pee Dee Belemnite, R=0.0112372. Atom % Excess (APE) is defined as Atom % (background) minus Atom % (sample). APE can be transferred to Mol % Excess (MPE) using the next formula:

$$\text{MPE} = \left[\frac{\text{APE}}{100 + \text{APE}} \times 100 \right]$$

Glutathione and Glycine measurement

Before analysis, erythrocytes were disrupted by freezing and thawing and subsequently sonicated for 5 min in an ultra sonification bath. Then 20 µL of 8 µmol/mL cycloleucine was added as internal standard to an aliquot of 200 µL erythrocyte solution and the samples were deproteinated by adding 100 µL of 2 M perchloric acid, incubated for 10 min on ice and finally centrifuged at 10000 x g for 20 min. The supernatant was transferred in a new tube and the pH was adjusted to 8 - 9 with approximately 20 µL of 4 M KOH. Excess perchloric acid was precipitated and removed by centrifugation at 10000 x g for 10 min. 50 µL of 1 M Na_2HPO_4 was added to maintain pH 9. The supernatant was filtered through 0.2 µm Nylon membrane filters (Grace Alltech, Breda, Netherlands). Then, 200 µL was transferred in a sample vial and 50 µL was injected for analysis of both glutathione concentration and ^{13}C- isotopic enrichment by LC-IRMS.

LC-IRMS conditions

Samples were introduced using a Midas autosampler (Spark) and analysed with a linear high-pressure gradient. Glycine and glutathione analyses (concentration and isotopic enrichment) were performed on a Sielc primesep A mixed mode column (250 × 3.2 mm, 5 µm), (Aurora Borealis, Schoonebeek,The Netherlands) at room temperature, (20 ± 2)°C. The LC flow rate was 500 µL-min^{-1}. The chromatographic separation is based on both ion exchange and hydrophobic interactions. After flushing the column with solvent A (**Table 1**) for 2.1 min, the LC gradient was increased in multiple steps from 0% to 80% using solvent B (1 M H_3PO_4, pH 2.2) (**Table 1**). Sodium peroxodisulfate 0.84 mol/L in sterile water served as oxidation solution. Acid reagent was prepared as 1.5 M phosphoric acid solution in sterile water. The flow rate of the acid and oxidant reagents in the LC interface were both 25 µL/min.

Table 1: LC-IRMS conditions for analysis of glycine and glutathione.

Analytical conditions						
Column	Sielc primesep A, 250x3.2mm, 5mm					
Solvent A	Milli-Q 18.2MW Water					
Solvent B	1M H_3PO_4					
Column temperature	20°C					
Column flow rate	0.5mL/min					
LC-IRMS parameters						
Reactor temperature	99.9°C					
Acid reagent	H_3PO_4, 1.5M, 25 ml					
Oxidant Reagent	$Na_2S_2O_8$, 0.8M, 25 ml					
Injection volume	25 ml					
Gradient profile used for GSSG analysis						
Time (min)	0	32	32.1	42	57	57.1
%B	0	0	10	80	80	0

Kinetic measurements

The fractional synthesis rate (FSR) is the percentage of the total renewal of a product per day. The incorporation of labelled glycine in glutathione was determined by measurement of the ^{13}C enrichment of glutathione in erythrocytes. The rate of incorporation of tracer is a reflection of the FSR of glutathione and was calculated according to the equation described below:

$$FSR = \frac{\text{slope } E_{[1\text{-}^{13}C]GSSGt4,5,6}}{E_{[1\text{-}^{13}C]glycine}} \times 24h \times 100\%$$

The ^{13}C isotopic enrichment of GSSG was measured by LC-IRMS. The slope $E_{[1\text{-}13C]GSSGt4,5,6}$ represents the increase / hour in isotopic enrichment of GSSG between 4 and 6 hours of infusion, expressed in Molar Percent Excess (MPE). $E_{[1\text{-}13C]glycine}$ represents the isotopic enrichment of intra-erythrocyte glycine, the precursor, in MPE at steady state.

Subsequently, the intravascular absolute synthesis rate of GSH (ASR_{GSH}) can be calculated using the following equation:

$$ASR_{GSH}(mg/(kg\bullet d)) = FSR_{GSH}/100 \times conc \times 307 \times ht \times 0.075$$

where conc is the GSH concentration in mmol/L of the erythrocyte fraction, 307 the molecular weight of GSH, ht the hematocrit content in L/L and 0.075 the estimated circulating blood volume in a neonate, expressed as L/kg.

Clinical study design

The study design was approved by the Erasmus MC Medical Ethical Review Board. The study population consisted of very low birth weight infants (birth weight <1500 g) admitted to the neonatal intensive care unit. Informed parental consent was obtained prior to the study. A primed (40 µmol/kg) continuous infusion of [1-^{13}C] glycine (20 µmol/(kg•h)) was administered intravenously for 6 hours. Blood samples were taken after 4, 5 and 6 hours (steady state). Portions of 400 µL freshly drawn EDTA blood were centrifuged at 900 x g for 10 min at 4°C. The upper layer was discarded and the lower layer – containing primarily erythrocytes and other cells – was reconstituted to the original volume with distilled water and stored at -80°C until further analysis.

RESULTS AND DISCUSSION

Chromatographic separation

Using this new LC-IRMS method, glycine and GSSG clearly stand out from the other eluting compounds, as shown in **Figures 1 and 2**. The retention times were stable with just a little variation in drift during the serial measurements: 29.6 +/- 0.5 min for glycine and 58.1 +/- 0.8 min for GSSG, respectively, evidence of robust chromatographic conditions. Cycloleucine was

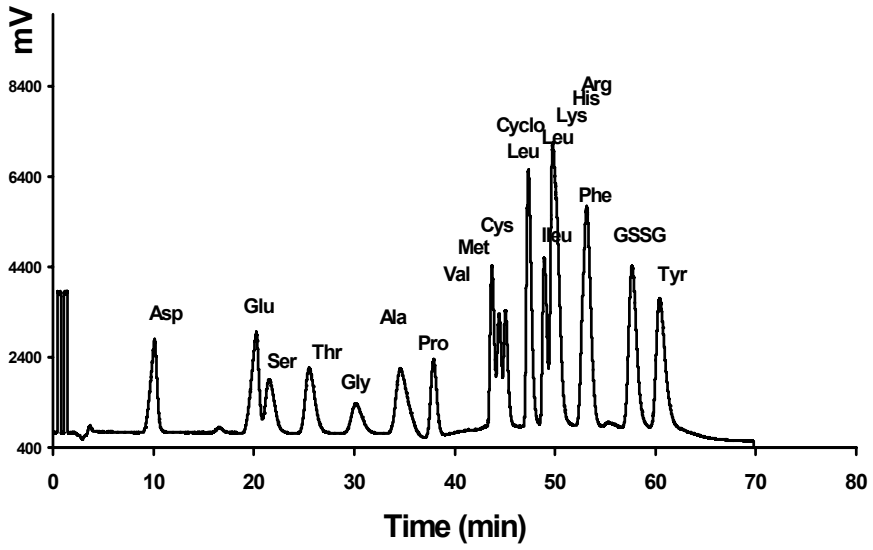

Figure 1: LC-IRMS chromatogram of 0.25 mmol hydrolysate amino acid standard mixed with 0.5 mmol glutathione standard solution.

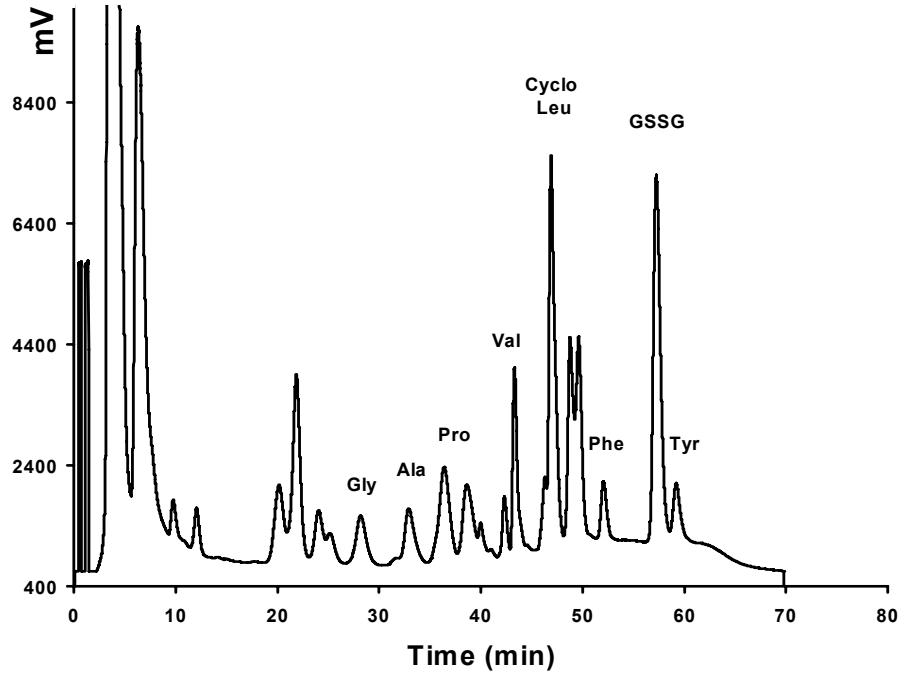

Figure 2: LC-IRMS chromatogram of glycine and glutathione in erythrocytes of human blood as its oxidized form GSSG.

chosen as internal standard because its chromatographic properties under the conditions used are better than those of e.g. norvaline and norleucine.

In our previous study we encountered the problem of glycine co-eluting with threonine and glutamate[28]. The use of a slightly different column, a Sielc primesep A instead of a primesep 100, and a modified gradient has solved this problem.

Additionally, in the method described here, several other amino acids, like threonine and aspartate, were nicely separated. This finding can be relevant to other neonatal studies dealing with stable isotopes.

Measurement of concentration of GSSG

The concentration of the tracee is an important parameter in every metabolic study. Therefore, a known amount of cycloleucine was added to each sample to be analyzed. This spiking of an internal standard (I.S.) made it possible to obtain the GSSG ratio versus I.S. and thus to assess GSSG concentrations in erythrocytes.

Five standard solutions of GSSG (using GSSG over I.S. area plotted against the mmol/L of GSSG injected) were measured between 0.1 to 2 mmol/L. A linear relationship was obtained ($y = 1.8081 + 0.004$). The regression coefficient (r^2) was calculated at 0.999 (**Figure 3**). The

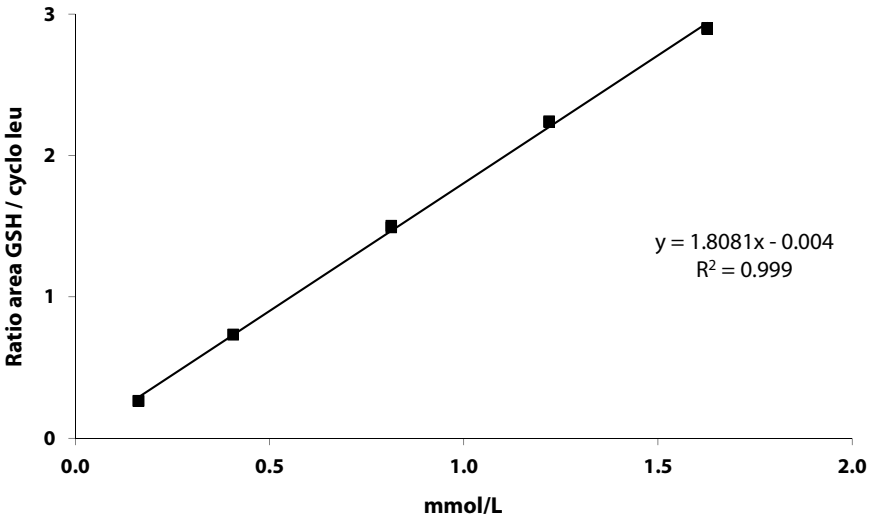

Figure 3: Calibration curve of glutathione concentration in human blood erythrocytes.

area of GSSG measured was between 15.7 ± 0.21 V.s and 189 ± 1.5 V.s. **Table 2** shows GSSG concentrations in erythrocytes per mL blood measured with only 150 µL of sample at three different time points in four subjects. The concentration of GSSG was measured with a good reproducibility (CV of 14.3%, when measured as duplicates). The mean value (0.54 ± 0.08 µmol/mL) of GSSG equivalent to the concentration of GSH was consistent with values reported in our previous paper[28] and in other literature[29]. These findings show that LC-IRMS can analyze the concentration of metabolites in blood with good precision (better than 0.1 µmol/mL) and a limit of detection (LOD) of 5 µmol/mL.

LC-IRMS measurement of glycine and GSSG ^{13}C enrichment in erythrocytes

After being oxidized, using the method described in the experimental section, an amino acid hydrolysate standard with a known added amount of GSH (**Figure 1**) as well as the erythrocyte samples (**Figure 2**) were analyzed using the LC-IRMS system. Because there is a little chromatographic shift in eluting time, when measuring ^{12}C /^{13}C, it is necessary to have a good separation of the compound of interest and other eluting compounds for a reliable determination of the ratio. As shown in **Figure 1 and 2**, it can be seen that glycine and GSSG were eluting in a part of the chromatogram free of other eluting compounds. The enrichment of ^{13}C glycine and ^{13}C GSSG was determined by comparing the ^{12}C /^{13}C ratio's using standard curves between 0% and 3% APE from known fractions of [1-^{13}C] glycine and [1,2-^{13}C- ^{15}N] glutathione. Linear relationships were obtained for glycine with a regression coefficient (R^2) calculated at 0.9999 (**Figure 4**) as well as for GSH with a regression coefficient (R^2) calculated at 0.9998 (**Figure 5**).

Table 2: Concentration and 13C- isotopic enrichments of glutathione in four different subjects.

Subject	Time (hour)	GSH mmol/mL	SD (n=2)	APE GSH	SD (n=2)
1	4a	0.49	0.007	0.214	0.000
	4b	0.48		0.215	
	5a	0.44	0.000	0.263	0.005
	5b	0.45		0.270	
	6a	0.44	0.003	0.346	0.000
	6b	0.44		0.345	
2	4a	0.54	0.007	0.065	0.008
	4b	0.53		0.076	
	5a	0.60	0.007	0.134	0.002
	5b	0.61		0.137	
	6a	0.58	0.002	0.191	0.004
	6b	0.58		0.186	
3	4a	0.45	0.018	0.166	0.005
	4b	0.47		0.160	
	5a	0.54	0.002	0.209	0.001
	5b	0.55		0.208	
	6a	0.48	0.007	0.270	0.001
	6b	0.49		0.271	
4	4a	0.65	0.022	0.155	0.002
	4b	0.62		0.152	
	5a	0.62	0.001	0.212	0.005
	5b	0.62		0.219	
	6a	0.61	0.004	0.260	0.003
	6b	0.61		0.255	
Mean		0.54	0.007	0.208	0.003
SD		0.077			
CV %		14.31			

Data are shown as mean, standard deviation (SD) and correlation variation (CV, %). Samples taken at 4, 5 and 6 hours were measured in duplicate.

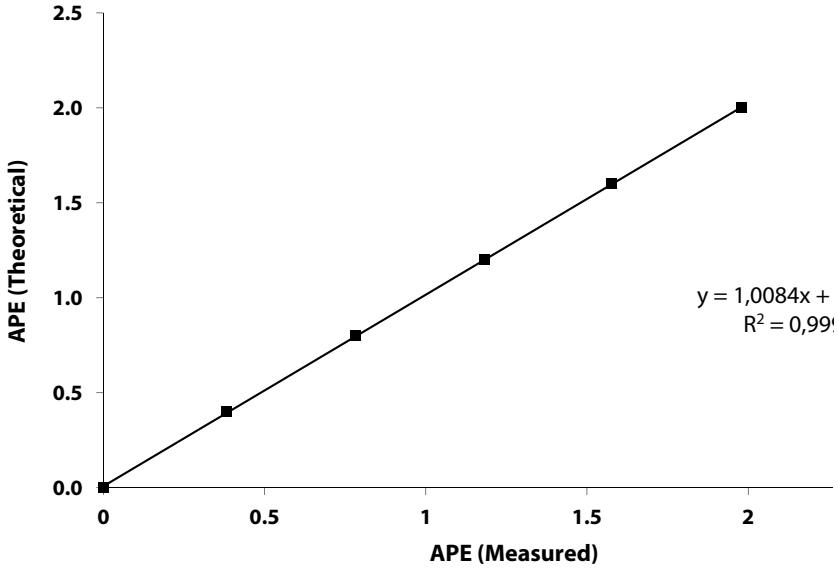

Figure 4: Calibration curve for measurement of [1-13C]-glutathione enrichment in human blood erythrocytes using [1,2-13C- 15N] glutathione.

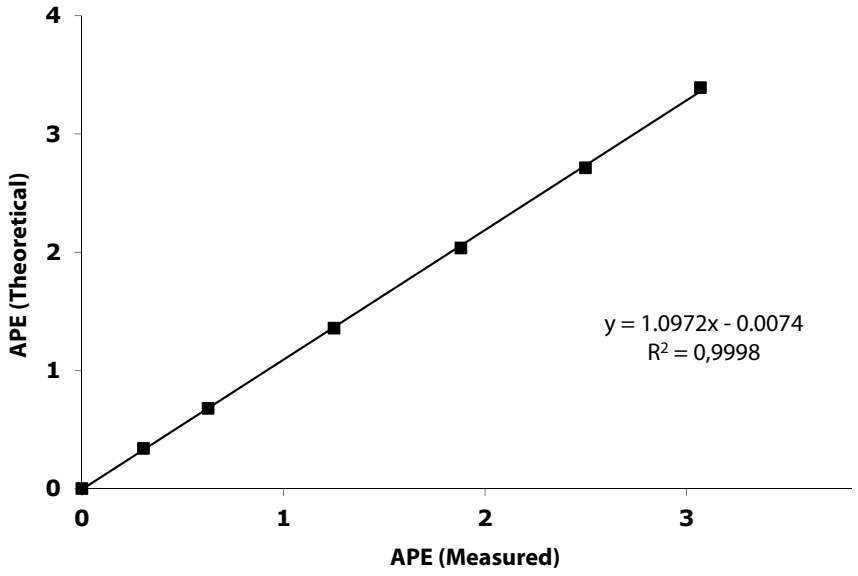

Figure 5: Calibration curve of 13C-glycine enrichment in human blood erythrocytes using [1-13C] glycine.

Accuracy and precision of isotopic measurement

As illustrated in **Figure 6**, the intra-assay repeatability assessed with cycloleucine measured with different blood samples from the same subject showed a sd of 0.09 measured at -27.03± 0.08 ‰ and a reproducibility (CV) of 0.4% (n=6). The inter-assay repeatability measured over different days was -27.01 ± 0.12 ‰ (n = 21). The CV was 0.8%. For each subject, the δ ^{13}C values of cycloleucine were close to the mean value. These values were in the same range as obtained with standard injection of I.S., evidence of excellent isotopic precision as well as accuracy of isotopic measurement at natural abundance.

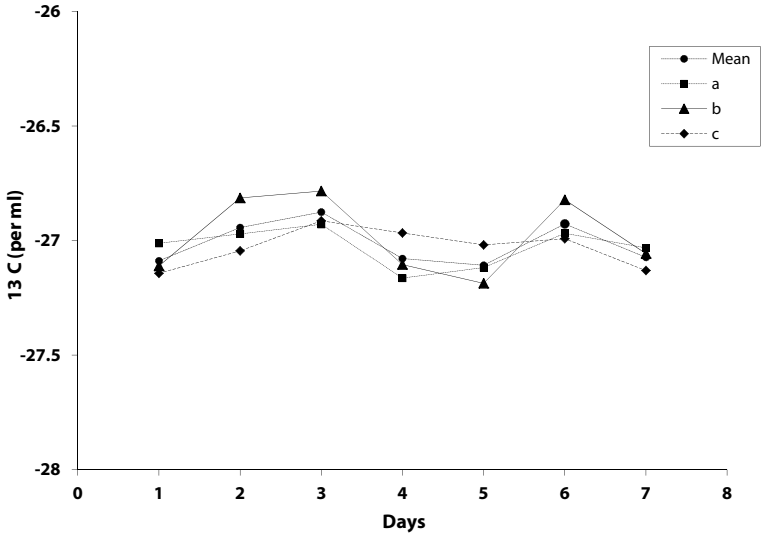

Figure 6: Variation of δ ^{13}C (‰) of cycloleucine added as internal standard and spiked in blood erythrocyte for 4 different subjects.
Blood was collected at three time points (a, b, c) and corresponding values are shown. Each point corresponds to duplicate injections.

The accuracy of isotopic measurement was assessed using standard calibration curve performed with various amounts of ^{13}C labeled GSH added to a fixed amount of natural GSH. By plotting measured APE versus theoretical APE between 0 and 2.5 APE, the curve was linear at a slope of 1.0084 (**Figure 4**). This shows that no isotopic fractionation occurred in the sample preparation and the analysis. The regression coefficient (R^2) was 0.9999, showing excellent linearity.

Calculation of the FSR of glutathione

During the steady state period of erythrocyte 1-^{13}C glycine levels, blood samples were taken at regular intervals (4h, 5h and 6h). The enrichments of glycine and GSH were determined in these erythrocyte samples. **Figure 7** shows a linear rise in time in GSSG APE. From these

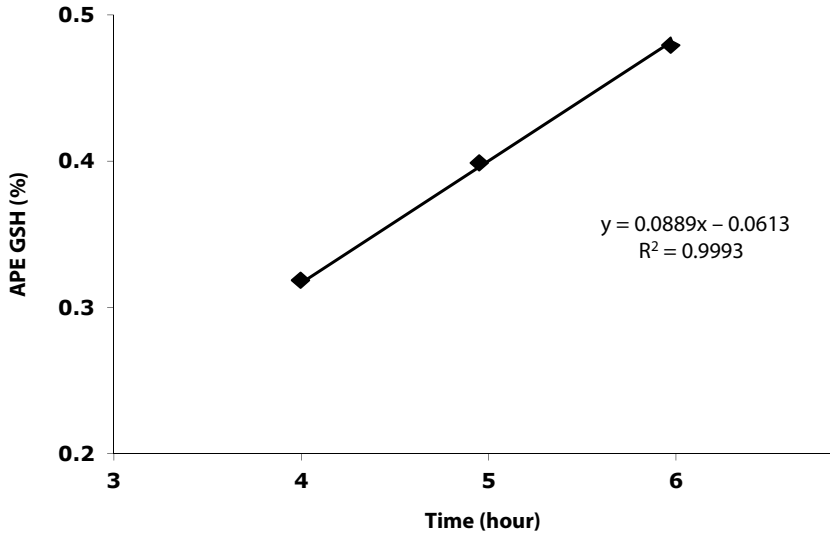

Figure 7: Graph of the increase of enriched glutathione of a subject expressed in APE / hour.

Table 3: The fractional synthetic rate (FSR %/d) of glutathione in four different subjects.

Subject	Glycine MPE	GSH APE %/Hour	GSH MPE %/Hour	GSH FSR %/day
1	3.80	0.0791	0.0733	46.27
2	3.73	0.0732	0.0682	43.86
3	4.07	0.0756	0.0703	41.43
4	3.59	0.0748	0.0696	46.53
Mean	3.80	0.0757	0.0703	44.53
SD	0.202	0.002	0.002	2.386
CV%	5.31	3.28	3.05	5.36

Glutathione (GSH) and glycine analyses were carried out in duplicate.

measurements FSR GSSG was calculated using the above equation 1. Values for four patients are reported in **Table 3**.

It would seem that the glutathione pool of these patients was renewed approximately every two days. FSR of glutathione was 44.5 ± 2.4%/day with a reproducibility (CV) of 5.4% (n = 4). These values were in the same range as reported previously[28].

CONCLUSION

This new LC-IRMS method for measuring kinetics of glutathione in its oxidized form (GSSG) shows to be a powerful tool in metabolic studies in neonates. Only little pre-purification is

necessary and the analyses reported here were fully automated. The simultaneous measurement of glycine and GSH, for both concentration and ^{13}C isotopic enrichment, gave excellent results. The more so as only 150 µL of blood was needed, which is of extremely high relevance for neonatal studies or studies in small animals. GSH concentrations corresponded to those reported in the literature. The precision and accuracy of isotopic enrichment at natural abundance and at higher isotopic enrichment gave excellent results without isotopic fractionation.

REFERENCES

1. Reid M, Badaloo A, Forrester T, Morlese JF, Frazer M, Heird WC, et al. In vivo rates of erythrocyte glutathione synthesis in children with severe protein-energy malnutrition. *Am J Physiol Endocrinol Metab* 2000;278(3):E405-12.
2. Meister A. Glutathione metabolism and its selective modification. *J Biol Chem* 1988;263(33):17205-8.
3. Lavoie JC, Spalinger M, Chessex P. Glutathione synthetic activity in the lungs in newborn guinea pigs. *Lung* 1999;177(1):1-7.
4. Jahoor F, Wykes LJ, Reeds PJ, Henry JF, del Rosario MP, Frazer ME. Protein-deficient pigs cannot maintain reduced glutathione homeostasis when subjected to the stress of inflammation. *The Journal of nutrition* 1995;125(6):1462-72.
5. Darmaun D, Smith SD, Sweeten S, Sager BK, Welch S, Mauras N. Evidence for accelerated rates of glutathione utilization and glutathione depletion in adolescents with poorly controlled type 1 diabetes. *Diabetes* 2005;54(1):190-6.
6. Deneke SM, Baxter DF, Phelps DT, Fanburg BL. Increase in endothelial cell glutathione and precursor amino acid uptake by diethyl maleate and hyperoxia. *Am J Physiol* 1989;257(4 Pt 1):L265-71.
7. Schaart MW, van der Schoor SRD, Schierbeek H, Stoll B, Burrin DG, van Goudoever JB. High intestinal utilization of threonine in piglets. *European Journal of Gastroenterology & Hepatology* 2005;17(1): A7-A8.
8. te Braake FWJ, Schierbeek H, de Groof K, Longini M, Buonocore G, van Goudoever JB. Glutathione kinetics and oxidative stress in pre term neonates in the first week of life. *Acta Paediatrica* 2007;96: 147-47.
9. Jahoor F, Jackson A, Gazzard B, Philips G, Sharpstone D, Frazer ME, et al. Erythrocyte glutathione deficiency in symptom-free HIV infection is associated with decreased synthesis rate. *Am J Physiol* 1999;276(1 Pt 1):E205-11.
10. Te Braake FW, Schierbeek H, de Groof K, Vermes A, Longini M, Buonocore G, et al. Glutathione synthesis rates after amino acid administration directly after birth in preterm infants. *Am J Clin Nutr* 2008; 88(2):333-9.
11. Riedijk MA, Stoll B, Chacko S, Schierbeek H, Sunehag AL, van Goudoever JB, et al. Methionine transmethylation and transsulfuration in the piglet gastrointestinal tract. *Proc Natl Acad Sci U S A* 2007; 104(9):3408-13.
12. Schaart MW, Schierbeek H, de Bruijn AC, Tibboel D, van Goudoever JB, Renes IB. A novel method to determine small intestinal barrier function in human neonates in vivo. *Gut* 2006;55(9):1366-7.
13. van den Akker CH, Schierbeek H, Dorst KY, Schoonderwaldt EM, Vermes A, Duvekot JJ, et al. Human fetal amino acid metabolism at term gestation. *Am. J. Clin. Nutr.* 2009;89(1):153-60.
14. Tea I, Ferchaud-Roucher V, Kuster A, Darmaun D, Robins RJ. Determination of 13C isotopic enrichment of glutathione and glycine by gas chromatography/combustion/isotope ratio mass spectrometry after formation of the N- or N,S-ethoxycarbonyl methyl ester derivatives. *Rapid Commun Mass Spectrom* 2007;21(20):3245-52.
15. Kuster A, Tea I, Sweeten S, Roze JC, Robins RJ, Darmaun D. Simultaneous determination of glutathione and cysteine concentrations and 2H enrichments in microvolumes of neonatal blood using gas chromatography-mass spectrometry. *Anal Bioanal Chem* 2008;390(5):1403-12.
16. Capitan P, Malmezat T, Breuille D, Obled C. Gas chromatographic-mass spectrometric analysis of stable isotopes of cysteine and glutathione in biological samples. *J Chromatogr B Biomed Sci Appl* 1999;732(1):127-35.
17. Schierbeek H, Berger R. Determination of succinylacetone and succinylacetoacetate in physiological samples as the common product 5(3)-methyl-3(5)-isoxazole propionic acid using an isotope dilution method and mass spectrometry. *Clin Chim Acta* 1989;184(3):243-50.

18. Schierbeek H, Bijsterveld K, Chapman TE, van Luijk WH, Reijngoud DJ, Berger R. Stable isotope dilution analysis of cystine in granulocyte suspensions as cysteine: a powerful method for the diagnosis, the follow-up, and treatment of patients with cystinosis. *Clin. Chim. Acta* 1990;191(1-2):39-47.
19. Cabanero AI, Recio JL, Ruperez M. Liquid chromatography coupled to isotope ratio mass spectrometry: a new perspective on honey adulteration detection. *Journal of agricultural and food chemistry* 2006;54(26):9719-27.
20. Heuer V EM, Tille S, Krummen M, Lollar XP, Hmelo LR, Hindrichs K. *Limnol. Oceangr. Methods* 2006;4: 346-57.
21. Smith CI, Fuller BT, Choy K, Richards MP. A three-phase liquid chromatographic method for delta13C analysis of amino acids from biological protein hydrolysates using liquid chromatography-isotope ratio mass spectrometry. *Analytical biochemistry* 2009;390(2):165-72.
22. Godin JP, Hau J, Fay LB, Hopfgartner G. Isotope ratio monitoring of small molecules and macromolecules by liquid chromatography coupled to isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2005;19(18):2689-98.
23. McCullagh JS, Juchelka D, Hedges RE. Analysis of amino acid 13C abundance from human and faunal bone collagen using liquid chromatography/isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2006;20(18):2761-8.
24. Godin JP, Fay LB, Hopfgartner G. Liquid chromatography combined with mass spectrometry for 13C isotopic analysis in life science research. *Mass Spectrom Rev* 2007;26(6):751-74.
25. Boschker HTS, Moerdijk-Poortvliet TCW, van Breugel P, Houtekamer M, Middelburg JJ. A versatile method for stable carbon isotope analysis of carbohydrates by high-performance liquid chromatography/isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2008;22(23):3902-8.
26. Godin JP, Breuille D, Obled C, Papet I, Schierbeek H, Hopfgartner G, et al. Liquid and gas chromatography coupled to isotope ratio mass spectrometry for the determination of 13C-valine isotopic ratios in complex biological samples. *J. Mass Spectrom.* 2008;43(10):1334-43.
27. McCullagh J, Gaye-Siessegger J, Focken U. Determination of underivatized amino acid delta(13)C by liquid chromatography/isotope ratio mass spectrometry for nutritional studies: the effect of dietary non-essential amino acid profile on the isotopic signature of individual amino acids in fish. *Rapid Commun Mass Spectrom* 2008;22(12):1817-22.
28. Schierbeek H, Te Braake F, Godin JP, Fay LB, van Goudoever JB. Novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2007;21(17):2805-12.
29. Shew SB, Jaksic T, Jahoor F, Heird WC. Effects of cysteine on endogenous synthesis of taurine and glutathione by premature neonates receiving parenteral nutrition. *Pediatric research* 2000;2000(47): 296A.

Part II

Oxidative stress at birth

Chapter 3

Resuscitation of very preterm infants with 30% vs. 65% oxygen at birth: study protocol for a randomized controlled trial

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ABSTRACT

Introduction

Resuscitation at birth with 100% oxygen is known to increase the oxidative burden with concomitant deleterious effects. Although fractions of inspired oxygen (FiO_2) < 100% are widely used in preterm infants, starting resuscitation at a (too) low FiO_2 may result in hypoxia. The objective of this study is to compare the safety and efficacy of resuscitating very preterm infants with an initial FiO_2 of 30% versus 65%.

Methods

In this double-blind, randomized controlled trial, 200 very preterm infants with a gestational age < 32 weeks will be randomized to start resuscitation after birth with either 30% or 65% oxygen. The FiO_2 will be adjusted based on oxygen saturation measured by pulse oximetry (SpO_2) and pulse rate (which should be over 100 beats per minute) in order to achieve a target SpO_2 of 88-94% at 10 min of life. The FiO_2 and pulse oximetry data will be continuously recorded.

The primary outcome is survival without bronchopulmonary dysplasia, as assessed by a physiological test at 36 weeks postmenstrual age. The secondary outcomes include the time to achieve SpO_2 > 88%, Apgar score at 5 min, cumulative O_2 exposure, oxidative stress (as determined by glutathione synthesis and oxidative stress markers), retinopathy of prematurity, brain injury and neurodevelopmental outcome at 2 years of age.

BACKGROUND

Resuscitating the newborn at birth with 100% oxygen is known to increase the oxidative burden with concomitant deleterious effects[1]. The latest International Liaison Committee on Resuscitation (ILCOR) guidelines recommend that "for babies born at term it is best to begin resuscitation with air rather than 100% oxygen" and that "administration of supplementary oxygen should be guided by oximetry"[2]. However, for preterm infants, the optimal fraction of inspired oxygen (FiO_2) to start resuscitation is still unknown. The ILCOR states that "blended oxygen and air may be given judiciously" and "both hyperoxemia and hypoxemia" should be avoided[2].

Several small studies on FiO_2 for resuscitating preterm infants have been performed. Wang et al. compared the use of initiating the resuscitation of preterm infants with either room air (*n = 18*) or 100% oxygen (*n = 23*)[3]. All infants in the room air group required an increase of the FiO_2 to achieve the targeted oxygen saturation (SpO_2), and the authors recommended that room air should not be used for resuscitating preterm infants. Escrig et al. compared initiating resuscitation of preterm infants with a gestational age (GA) ≤ 28 weeks with either 30% or 90% oxygen[4]. In this study, the FiO_2 in the low-oxygen group (*n = 19*) was increased stepwise to 45%, and the FiO_2 in the high-oxygen group (*n = 23*) was reduced to 45% to reach the target SpO_2. In a similar study by Vento et al., resuscitation with 30% oxygen (n = 37) resulted in decreased oxidative stress markers and a decreased risk of bronchopulmonary dysplasia (BPD) compared to starting resuscitation with 90% (n = 41)[5]. Also in this study, FiO_2 in both groups was increased stepwise in the low-oxygen group and decreased in the high-oxygen group reaching 55% at 5 min in both groups.

From these data, it can be concluded that initiating the resuscitation of preterm infants with room air is too low, while starting with 90% FiO_2 is too high. Because it is important to avoid both hypoxia and hyperoxia, the optimal initial FiO_2 for resuscitating preterm infants needs to be determined. Therefore, we hypothesize that resuscitation of very preterm infants (GA < 32 weeks) with an initial FiO_2 of 30% is safe, decreases oxidative stress and improves outcome compared to resuscitation with an initial FiO_2 of 65%.

DESIGN

Trial Design

The study is a double-blind, randomized controlled trial and will be performed in the Neonatal Intensive Care Unit (NICU) of the Erasmus MC - Sophia Children's Hospital, Rotterdam, the Netherlands. The study is investigator-initiated, without funding from the pharmaceutical industry. The study protocol has been approved by the Erasmus MC Medical Ethics Committee. Serious adverse events (death, retinopathy of prematurity (ROP) ≥ grade 3 and intraventricular hemorrhage (IVH) ≥ grade 3) will be reported to the medical ethics committee, which will monitor the study safety.

Subjects

The inclusion criteria are infants with a GA < 32 weeks born at the Erasmus MC - Sophia Children's Hospital. Assessment of the GA will be based on early fetal ultrasonography or on the date of last menstrual period. The exclusion criteria are any known major congenital malformations, chromosome defects, or metabolic, endocrine or renal disorders. Because this study involves an acute intervention at birth, written informed consent will be obtained antenatally. All mothers admitted to Erasmus MC-Sophia Children's Hospital who are at risk for preterm delivery before 32 weeks of gestation (e.g. premature labor, preeclampsia, intrauterine growth retardation) will be approached for participation in the study. When parents have consented to participate in the study and there has been an actual preterm delivery, the preterm infant will be included at birth.

Research setting and randomization

The Erasmus MC - Sophia Children's Hospital has six resuscitation units, which have been modified for this study by adding an additional oxygen blender (PM5200, Precision Medical Inc., Northampton, PA, USA) (**Figure 1**). This additional oxygen blender is not visible to the physician and will be randomized after each inclusion to either 30% or 65% oxygen using a computer generated list. When an infant with prenatal consent is born, the physician will activate the research setting by activation of a switch just before delivery. By activating this research switch, the regular oxygen blender (Bird Ultrablender, Cardinal Health, Dublin, OH, USA) will be connected to the additional oxygen blender. Administered oxygen will come from the additional oxygen blender, randomized to 30% or 65% oxygen, and thus resuscitation will be started with either 30% or 65% oxygen.

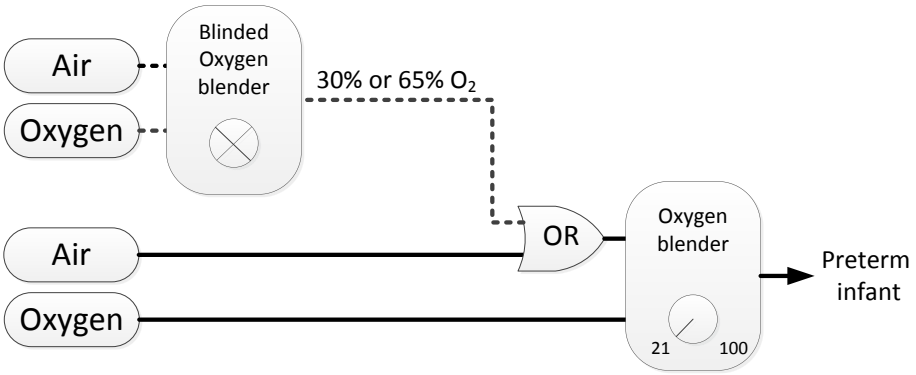

Figure 1: Adjusted resuscitation unit.
Dotted lines depict the adjustments made to the resuscitation unit. The blinded oxygen blender is randomized to administer either 30% or 65% oxygen. The OR-port depicts the switch by which the research setting can be activated or deactivated.

Resuscitation

All resuscitations of preterm infants are performed by a neonatologist or an experienced neonatologist in training. Immediately after cord clamping, the infant will be placed on the resuscitation unit. The resuscitation is performed according to standard guidelines, i.e. the infant is stimulated and heat loss is prevented. A disposable SpO_2 sensor (Nellcor Max-N, Covidien, Dublin, Ireland) will be applied to the right hand or wrist before switching on the pulse oximeter (Nellcor OxiMax N-600x, Covidien, Dublin, Ireland). Infants will be resuscitated with either a flow inflating mask (Jackson Reese modification T-piece system breathing system, Intersurgical, Wokingham, UK) or a T-piece resuscitator (Neopuff, Fisher & Paykel Healthcare, Auckland, New Zealand), according to the physician's preferences.

Resuscitation is started with either 30% or 65% oxygen, for which the physician will be blinded. The objective of the resuscitation is to achieve a target SpO_2 of 88-94% at 10 min after birth. If the pulse rate remains stable and over 100 beats per minute (bpm), no adjustment of FiO_2 is advised. At all times, the physician can adjust the FiO_2 if the clinical situation is not satisfactory (e.g. persistent bradycardia or SpO_2 > 94%). To adjust the FiO_2, the physician deactivates the research switch by which the additional oxygen blender is disconnected and the administered FiO_2 is supplied via the regular oxygen blender. The FiO_2 can then be manually adjusted to the desired FiO_2, without the physician being aware of the initial FiO_2 to which the infant was randomized.

Outcome parameters

Primary outcome

Survival without BPD at 36 weeks postmenstrual age (PMA).

Secondary outcomes

1. Resuscitation: Apgar score at 5 min, time after birth to achieve SpO_2 > 88% and cumulative O_2 exposure during resuscitation.
2. Oxidative stress: Glutathione (GSH) synthesis and oxidative stress markers.
3. Incidence of ROP and brain injury.
4. Neurodevelopmental outcome at 2 years of age.

The timeline of the study design is depicted in **Figure 2**.

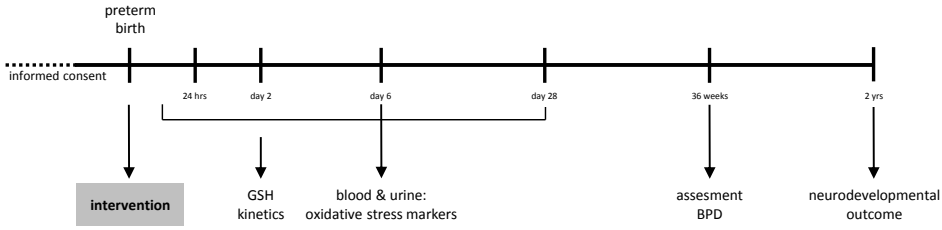

Figure 2: Timeline of the study design.
GSH = glutathione, BPD = bronchopulmonary dysplasia.

Collection resuscitation data

Medical record documentation often varies from actual interventions, especially in acute situations[6]. Therefore, video recordings will be made to analyze resuscitation with regard to time of birth and the timeline of the resuscitation (e.g. time to start SpO_2 recording, time to start respiratory support and the time to intubation, when applicable). The time of birth is defined as the time of cord clamping. The FiO_2 will be continuously recorded (1 Hz) using a medical oxygen monitor (MX300 Medical Oxygen Monitor, Teledyne, City of Industry, CA, USA). The SpO_2 will be continuously recorded (0.5 Hz) using serial port reader software (TeraTerm, Open Source Software).

Clinical definitions

Bronchopulmonary Dysplasia

Two definitions for the diagnosis of BPD will be used. First, the definition of BPD for preterm infants with a GA < 32 weeks described by Jobe et al. will be used (**Table 1**)[7]. According to this definition, BPD is present when an infant is treated with FiO_2 > 21% for at least 28 days and is further classified according to the need for oxygen and/or respiratory support at 36 weeks PMA.

Table 1: Criteria for bronchopulmonary dysplasia for preterm infants with a gestational age < 32 weeks.

Treatment with oxygen >21% for at least 28 days **plus**	
Mild BPD	Breathing room air at 36 weeks PMA
Moderate BPD	Need for < 30% oxygen at 36 weeks PMA
Severe BPD	Need for ≥ 30% oxygen and/or positive pressure at 36 weeks PMA

Adapted from Jobe et al.[7]. BPD = bronchopulmonary dysplasia, PMA = postmenstrual age. A day of treatment with oxygen > 21% means that the infant received oxygen > 21% for more than 12 h on that day.

Second, the diagnosis of BPD will be assessed at 36 weeks PMA based on the physiological criteria of Walsh et al.[8]. Infants treated with mechanical ventilation or continuous positive airway pressure (CPAP), or infants receiving FiO_2 ≥ 30% oxygen with oxygen saturations < 96% are diagnosed with BPD. For infants receiving < 30% oxygen or infants receiving ≥ 30% oxygen with oxygen saturations > 96%, a timed oxygen reduction test will be performed as described by Walsh et al.[8]. During the timed oxygen reduction test, BPD is diagnosed when oxygen saturations are < 90% for more than 5 consecutive min or < 80% for more than 15 s. No BPD is defined as oxygen saturations ≥ 90% during weaning to room air.

Retinopathy of prematurity

As part of standard care, ROP will be assessed at a postnatal age of 5 weeks by a pediatric ophthalmologist. ROP will be diagnosed and classified according to the International Classification of Retinopathy of Prematurity (ICROP)[9].

Brain injury

Substantial brain injury will be diagnosed according to the definition previously described in the EUNO trial[10], i.e. grade 3 or 4 IVH[11] or periventricular leukomalacia based on ultrasound images and/or MRI of the brain[12].

Neurodevelopmental outcome

As part of standard care, all preterm infants born with a GA < 32 weeks will be followed until 2 years of age. At 2 years, certified neurodevelopmental physiologists will evaluate the infants using the Bayley Scales of Infant Development 3rd Edition (BSID III).

Glutathione concentration and synthesis rates

Tracer infusion protocol and blood sampling

On the second postnatal day, a primed (20 µmol/kg) continuous (20 µmol/(kg•h)) infusion of [U-^{13}C]glycine (99% enriched, Cambridge Isotope Laboratories, Andover, MA, USA; sterility and pyrogenicity tested) will be administered during 8 h using a Perfusor fm infusion pump (B|Braun Medical B.V., Oss, The Netherlands). Blood will be sampled from an indwelling arterial catheter after 6, 7 and 8 h and collected in EDTA containing microtainers. After centrifugation at 3500 x g for 10 min, the plasma fraction will be removed, and the lower layer (containing primarily erythrocytes) will be reconstituted to its original volume with ice-cold distilled water. To calculate the fractional synthesis rates (FSR) and absolute synthesis rates (ASR) of GSH, concentration and enrichment of GSH and its precursor glycine will be determined in the erythrocytes.

Glutathione enrichment and concentration

Analysis of GSH will be performed on a LC-Isolink interface coupled to a Delta XP isotope ratio mass spectrometer (LC-IRMS) (Thermo Fisher, Bremen, Germany) using a recently developed method[13]. This highly sensitive method requires only a very small sample and does not require derivatization of the sample.

Glycine enrichments

The erythrocyte enrichment of ^{13}C glycine will be measured by gas chromatography mass spectrometry (GCMS) as its ethyl chloroformate (ECF) ester derivatives, using a MSD 5975C Agilent GCMS (Agilent Technologies, Amstelveen, The Netherlands). Briefly, 25 µl aliquots of the remaining supernatant used for the GSH analysis will be acidified by adding 50 µl of 0.1 M HCl and diluted with 125 µl of distilled water. ECF derivatization of the samples will be performed according to a modified procedure of Hušek[14]. A CP-Sil 17 column (25 m x 0.25 mm id, 0.12 µm film thickness; Varian, Middelburg, the Netherlands) will be used for the separation. The samples will be measured using a selected ion monitoring mode (SIM) method. The mass fragments with a mass to charge (*m/z*) of 102.1 for unenriched (*M*) and an *m/z* 103.1 for the enriched (*M* + 1) glycine respectively, are selected for this purpose.

Calculations

The FSR_{GSH} represents the fraction of the total intraerythrocytic GSH pool that is renewed per unit of time and is expressed as %/d.

$$FSR_{GSH}\,(\%/d) = \frac{\text{slope } E_{[U\text{-}^{13}c]GSH_{t6,7,8}}}{E_{\text{intraerythrocystic}[U\text{-}^{13}c]\text{glycine}}} \times 24h \times 100\%$$

where E stands for the enrichment expressed as MPE. The nominator (product) of this equation represents the hourly increase in [U-^{13}C]glycine bound to GSH, as calculated from the increase in enrichment between 6 and 8 h of infusion. The denominator (precursor) represents the intraerythrocytic U-^{13}C enrichment of free glycine at isotopic steady state.

Subsequently, the intravascular ASR_{GSH}, which represents the absolute amount of GSH that is produced per unit of time (mg/(kg·d)), can be calculated using the following equation:

$$ASR_{GSH}\,(mg/(kg \bullet d) = FSR_{GSH}/(100 \times conc. \times 307 \times ht \times 0.075),$$

where conc. is GSH concentration in mmol/l packed erythrocytes, 307 is the molecular weight of GSH, ht is hematocrit and 0.075 is the estimated circulating volume in a preterm neonate, expressed as L/kg.

Oxidative stress markers

Oxidative stress markers in plasma and urine will be determined within 24 h of birth, on postnatal day 6 and on postnatal day 28.

Non protein bound iron (NPBI)

Blood samples, drawn from an arterial catheter or via a heel prick, will be collected in heparinized microtainers and immediately placed on melting ice. After centrifugation at 3500 x g for 10 min, the plasma fraction will be removed from the lower layer and stored at -80°C until further analysis. Plasma samples will be shipped on dry ice to the University of Siena (Siena, Italy), where the NPBI will be determined according to previously described methods[15].

Isoprostanes and 8-hydroxy-2'-deoxyguanosine (8-oxo-dG)

Urine will be collected by placing gauzes in the infants' diapers. After centrifugation at 2800 x g for 5 minutes, the urine will be stored at -80°C until further analysis. Urine samples will be shipped on dry ice to the University Hospital LA Fe (Valencia, Spain), where the urinary isoprostane and 8-oxo-dG will be determined according to methods previously described[16].

Statistical analysis

Power calculation based on the incidence of BPD shows that, with an incidence of 30% and an expected reduction of 15%, 100 infants per group will be needed to find a statistically significant difference with an α of 0.05 and a power of 0.80. Differences between groups will be assessed using the Mann-Whitney test for continuous measurements and the chi-square test for categorical measurements ($p < 0.05$).

DISCUSSION

Although this study concerns the acute intervention of neonatal resuscitation, the randomization and blinding are optimal in this study design. Because cases of acute preterm delivery will not be included in this study, the main limitation will be the selection bias. Since informed consent will be obtained before birth, only mothers who are actually hospitalized antenatally will be approached by the researchers. Consequently, all included infants will have received at least one dose of prenatal steroids. As prenatal steroids have proven to be beneficial to immature lungs, included infants will likely have less respiratory difficulties than the acute cases. Furthermore, administration of prenatal steroids is associated with increased antioxidant enzyme activity, which reduces susceptibility to hypoxia and to oxidative damage as a result of hyperoxia[17]. In short, the studied cases may show less morbidity such as BPD than the total population of very preterm infants.

The selection bias could be circumvented by a waiver of informed consent. In 1996, the Food and Drug Administration (FDA) and the Department of Health and Human Services (DHHS) published guidelines on exceptions from the informed consent requirements in specific situations[18]. For emergency research, these guidelines stipulate that the institutional review board (IRB) may approve a clinical investigation without requiring informed consent from all research subjects after meeting certain criteria. These criteria include informed consent not being feasible because the intervention must be performed before consent from the subjects' legally authorized representative can be obtained, as would be the case in this study when preterm infants are born acutely. Because informed consent can be sought in a sufficient number of very preterm deliveries, we decided that it would not be ethical to use a waiver of informed consent.

This study is performed in an affluent healthcare setting and blended oxygen might not be available in all hospitals. However, according to international guidelines, preterm infants should be born in a tertiary hospital whenever possible and, in Western countries, most very preterm infants are indeed born within a tertiary hospital. Since it has been shown that room air and 100% oxygen are both not ideal in the resuscitation of preterm infants, it is important to study the optimal FiO_2 to start resuscitation of these infants.

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REFERENCES

1. Saugstad OD, Ramji S, Soll RF, Vento M. Resuscitation of Newborn Infants with 21% or 100% Oxygen: An Updated Systematic Review and Meta-Analysis. *Neonatology* 2008;94(3):176-82.
2. Perlman JM, Wyllie J, Kattwinkel J, Atkins DL, Chameides L, Goldsmith JP, et al. Part 11: neonatal resuscitation: 2010 International Consensus on Cardiopulmonary Resuscitation and Emergency Cardiovascular Care Science With Treatment Recommendations. *Circulation* 2010;122(16 Suppl 2): S516-38.
3. Wang CL, Anderson C, Leone TA, Rich W, Govindaswami B, Finer NN. Resuscitation of preterm neonates by using room air or 100% oxygen. *Pediatrics* 2008;121(6):1083-9.
4. Escrig R, Arruza L, Izquierdo I, Villar G, Saenz P, Gimeno A, et al. Achievement of targeted saturation values in extremely low gestational age neonates resuscitated with low or high oxygen concentrations: a prospective, randomized trial. *Pediatrics* 2008;121(5):875-81.
5. Vento M, Moro M, Escrig R, Arruza L, Villar G, Izquierdo I, et al. Preterm Resuscitation With Low Oxygen Causes Less Oxidative Stress, Inflammation, and Chronic Lung Disease. *Pediatrics* 2009;124(3):e439-49.
6. Rich WD, Leone T, Finer NN. Delivery room intervention: improving the outcome. *Clinics in perinatology* 2010;37(1):189-202.
7. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 2001;163(7):1723-9.
8. Walsh MC, Yao Q, Gettner P, Hale E, Collins M, Hensman A, et al. Impact of a physiologic definition on bronchopulmonary dysplasia rates. *Pediatrics* 2004;114(5):1305-11.
9. The International Classification of Retinopathy of Prematurity revisited. *Arch Ophthalmol* 2005;123(7): 991-9.
10. Mercier JC, Hummler H, Durrmeyer X, Sanchez-Luna M, Carnielli V, Field D, et al. Inhaled nitric oxide for prevention of bronchopulmonary dysplasia in premature babies (EUNO): a randomised controlled trial. *Lancet* 2010;376(9738):346-54.
11. Papile LA, Burstein J, Burstein R, Koffler H. Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. *J Pediatr* 1978;92(4): 529-34.
12. De Vries LS, Regev R, Pennock JM, Wigglesworth JS, Dubowitz LM. Ultrasound evolution and later outcome of infants with periventricular densities. *Early Hum Dev* 1988;16(2-3):225-33.
13. Schierbeek H, Te Braake F, Godin JP, Fay LB, van Goudoever JB. Novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2007;21(17):2805-12.
14. Husek P. Rapid derivatization and gas chromatographic determination of amino acids. *Journal of Chromatography* 1991;552:289-99.
15. Paffetti P, Perrone S, Longini M, Ferrari A, Tanganelli D, Marzocchi B, et al. Non-protein-bound iron detection in small samples of biological fluids and tissues. *Biol Trace Elem Res* 2006;112(3):221-32.
16. Milne GL, Sanchez SC, Musiek ES, Morrow JD. Quantification of F2-isoprostanes as a biomarker of oxidative stress. *Nat Protoc* 2007;2(1):221-6.
17. Vento M, Aguar M, Escobar J, Arduini A, Escrig R, Brugada M, et al. Antenatal steroids and antioxidant enzyme activity in preterm infants: influence of gender and timing. *Antioxidants & redox signaling* 2009;11(12):2945-55.
18. Administration FaD, Services DoHaH. Protection of human subjects., 1996.

Chapter 4

No differences in clinical outcome or oxidative stress between an initial fraction of inspired oxygen of 30% and 65% during resuscitation of preterm infants after birth: a double-blind, randomized controlled trial

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Submitted

ABSTRACT

Introduction

Resuscitation at birth with 100% oxygen is associated with increased mortality and morbidity in term infants. However, no evidence-based guidelines on the initial fraction of inspired oxygen (FiO_2) for resuscitation of preterm infants are available. We hypothesised that an initial FiO_2 of 30% during resuscitation of preterm infants after birth results in less oxidative stress and improves clinical outcome compared to 65% oxygen.

Methods

In a double-blind, randomized trial, preterm infants with a gestational age (GA) <32 weeks were randomized to start oxygen supplementation after birth with either 30% (low-oxygen group) or 65% oxygen (high-oxygen group) after which the FiO_2 was adjusted based on the oxygen saturation measured by pulse oximetry (SpO_2). Primary outcome was survival without bronchopulmonary dysplasia (BPD) assessed at 36 weeks postmenstrual age. Secondary outcomes included were markers of oxidative stress. Data are presented as median (interquartile range)

Results

A total of 193 infants with a median GA of 28⅘ (26⅖ - 30⅗) weeks were included. Survival without BPD was not different between the low-oxygen and high-oxygen group (72% and 75% respectively, p=0.75). The FiO_2 in both groups was adjusted to mean 40% by seven minutes (low-oxygen, interquartile range 30-42%) and 11 minutes (high-oxygen, interquartile range 21-53%) after which the FiO_2 remained similar during the entire time in the delivery room. No differences were found for markers of oxidative stress.

Conclusions

Initial supplementation of preterm infants with 30% oxygen during foetal-to-neonatal transition is as safe as 65%. Because the oxygen load was lower in the low-oxygen group, we advise to start the resuscitation of preterm infants with an initial FiO_2 of 30% and adjusting it according to recent SpO_2 guidelines.

INTRODUCTION

Approximately 5-10% of newborn infants require some assistance to establish breathing after birth, which can be achieved by simple warming, drying, and stimulation for most term infants[1]. Resuscitation of newborn infants at birth with 100% oxygen is known to increase the oxidative burden with a concomitant increase in morbidity and mortality. Therefore the use of 100% oxygen has become obsolete for the resuscitation of term infants[2 3]. During resuscitation, the majority of preterm infants need active respiratory support[4] with, in most cases, administration of oxygen. Recently, a nomogram of oxygen saturation measured by pulse oximetry (SpO_2) obtained from infants who did not need intervention in the delivery room was published[5]. Although not tested in randomized trials in preterm infants, up to date this seems the best reference to guide oxygen supplementation during resuscitation[6 7]. However, the optimal fraction of inspired oxygen (FiO_2) to start resuscitation is still unknown. The latest International Liaison Committee on Resuscitation (ILCOR) guidelines state that "both hyperoxemia and hypoxemia" should be avoided without an advice on the initial FiO_2[8].

Several small studies on FiO_2 for the resuscitation of preterm infants have been performed comparing a low and high initial FiO_2[9-11]. In these studies, FiO_2 in the low-oxygen group was increased upwards and FiO_2 in the high-oxygen group was decreased downwards. In the study by Vento et al., resuscitation of extremely preterm infants with a gestational age (GA) ≤ 28 weeks gestation with an initial FiO_2 of 30% (n = 37) resulted in decreased oxidative stress markers and a decreased risk of bronchopulmonary dysplasia (BPD) compared to an initial FiO_2 of 90% (n = 41)[11].

From these data, it could be concluded that initiating the resuscitation of preterm infants with room air may be too low, while starting with 90% FiO_2 is too high. Because both hypoxia and hyperoxia during resuscitation can have detrimental effects, the optimal initial FiO_2 for resuscitating preterm infants needs to be determined. Compared to a high FiO_2, an initial FiO_2 of 30% is proven to be beneficial. However, to assess the safety and efficacy of starting resuscitation of preterm infants with low oxygen concentrations, the next objective was to compare 30% oxygen with an intermediate FiO_2. We hypothesised that assisting very preterm infants (GA < 32 weeks) during foetal-to-neonatal transition with an initial FiO_2 of 30% would be safer, would decrease oxidative stress, and improve outcome compared to the use of an initial FiO_2 of 65%.

SUBJECTS AND METHODS

This double-blind, randomized controlled trial was performed on the neonatal intensive care unit (NICU) of the Erasmus MC - Sophia Children's Hospital (Rotterdam, the Netherlands), a level III unit. The study protocol was approved by the Erasmus MC Medical Ethics Committee.

Subjects

Preterm infants with a GA < 32 weeks born at the Erasmus MC - Sophia Children's Hospital were eligible for this study. Assessment of GA was based on early foetal ultrasonography or on the date of last menstrual period. Written informed consent was obtained from all parents antenatally, because the intervention was performed directly after birth. Mothers at risk for preterm delivery before 32 weeks of gestation were approached for participation in the study. Exclusion criteria included major congenital malformations, chromosome defects, and metabolic or endocrine disorders.

Randomisation and masking

A full description of the study design and resuscitation procedures have been previously reported[12]. In short, the resuscitation units were modified with an additional oxygen blender (PM5200, Precision Medical Inc., Northampton, PA, USA). Before the start of resuscitation, this additional oxygen blender was activated by a switch on the resuscitation unit. When activated, resuscitation started randomly with either 30% oxygen (low-oxygen group) or 65% oxygen (high-oxygen group) for which the physician was blinded. The objective of the resuscitation was to achieve a target SpO_2 of 88-94% at ten minutes after birth. If the pulse rate remained stable and over 100 beats per minute (bpm), no intervention was advised. At all times, the physician could adjust the FiO_2 if the clinical situation was not satisfactory (e.g., persistent bradycardia or SpO_2 >94%). To adjust the FiO_2, the physician deactivated the research switch, which deactivated the additional oxygen blender. Subsequently, the FiO_2 was supplied via the regular oxygen blender and could be manually adjusted to the desired FiO_2, without knowledge of the initial FiO_2 to which the infant was randomized.

Outcome parameters

The primary outcome was survival without BPD at 36 weeks postmenstrual age (PMA), diagnosed according to the physiological criteria described by Walsh et al., including room-air challenge tests at 36 weeks postmenstrual age[13]. Secondary outcomes included the Apgar score at 5 minutes, time after birth to achieve a SpO_2 > 88%, incidence of retinopathy of prematurity (ROP) and brain injury, and oxidative stress. Clinical definitions have been described previously[12]. Oxidative stress was determined in subgroups by measurement of glutathione (GSH) concentration and synthesis, plasma non protein bound iron (NPBI) concentration, and urinary oxidative stress markers. The timeline of the intervention and outcome parameters are depicted in **Figure 1**.

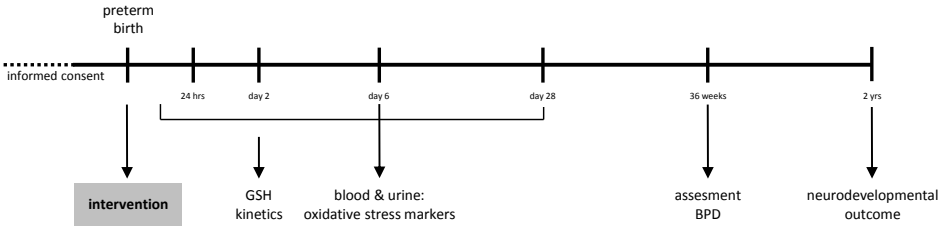

Figure 1: Timeline of outcome parameters.
GSH = glutathione, BPD = bronchopulmonary dysplasia. Reprinted from Rook et al.[12].

Oxidative stress

GSH concentration and synthesis rates were determined on postnatal day two using a stable isotope study according to methods previously described[12].

NPBI concentration was determined within 24 hours after birth and on day 6. Blood samples were collected in heparinised microtainers and immediately placed on melting ice. After centrifugation at 3500 x g for ten minutes, the plasma fraction was removed from the lower layer and stored at -80°C until further analysis. Plasma samples were shipped on dry ice to the University of Siena (Siena, Italy), where the NPBI was determined according to methods previously described[14].

Urinary oxidative stress markers were determined within 24 hours after birth and on day 6. Urine was collected by placing gauze in the infants' diapers. After centrifugation at 2800 x g for 5 minutes, the urine was stored at -20°C until further analysis. Urine samples were shipped on dry ice to the University & Polytechnic Hospital La Fe (Valencia, Spain), where the urinary 8-hydroxy-2'-deoxyguanosine/2-deoxyguanosine ratio (8OhdG/2dG; a marker of oxidative damage to DNA), ortho-tyrosine/phenylalanine ratio (O-Tyr/Phenyl; a marker of oxidative damage to protein, 3-nitro-tyrosine (N-Tyr; a marker of nitrosative damage to protein), and 3-chlor-tyrosine (Cl-Tyr; a marker of inflammation caused by free radicals) were determined by high performance liquid chromatography coupled to mass spectrometry[15], which was modified for 3-N-Tyr and 3-Cl-Tyr based on the study by Orhan et al.[16].

Statistics

Power calculation based on the incidence of BPD showed that, with an incidence of 30% (based on cohort studies[13 17]) and an expected reduction of 15% using a 1-sided test, 100 infants per group were needed to find a statistically significant reduction with an α of 0.05 and a power of 0•80. Differences between groups were assessed using the Mann-Whitney test for continuous measurements and the Chi-square test for categorical measurements ($p < 0.05$). All analyses were performed according to an intention-to-treat principle.

RESULTS

From a total of 781 eligible preterm infants, 193 were analysed in the study (**Figure 2**). The main reason for missed inclusion was imminent preterm delivery, which left no time to obtain

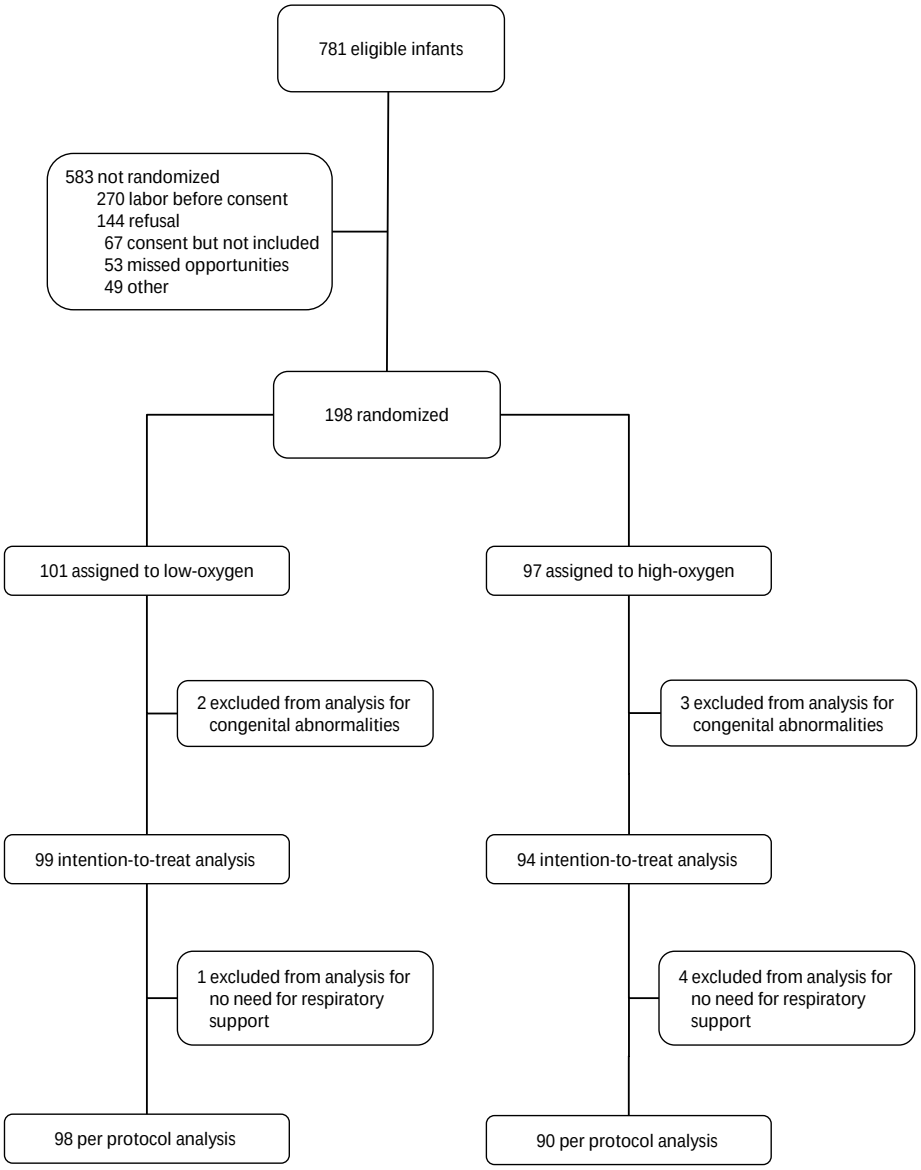

Figure 2: Trial profile.

Table 1: Clinical and obstetric baseline characteristics.

	Low-oxygen initial FiO_2 30%, n=99	**High-oxygen** initial FiO_2 65%, n=94
Female	55 (56%)	51 (54%)
Gestational age (weeks)	28⅚ (27⅙ - 30⅗)	29⅔ (26⅗ - 30⅘)
Birth weight (g)	1013 (820 - 1280)	1123 (790 - 1368)
Umbilical cord pH	7.31 (7.27 - 7.34)	7.30 (7.25 - 7.34)
CRIB score#	2 (1 - 7)	1 (1 - 5)
Prenatal corticosteroids (%)	100%	100%
Incomplete course (%)	7%	11%
Type of delivery (vaginal: CS)	31 : 68	33 : 61

Data are presented as median (interquartile range) or n (%).
The CRIB (Clinical Risk Index for Babies) indicating the degree of illness[18]. CS = caesarean section.

informed consent prior to birth. Due to the acute character of the resuscitation of preterm infants, 67 infants were missed despite consent. After randomization, 5 infants were excluded due to postnatally diagnosed major congenital abnormalities. General demographic and obstetric characteristics are depicted in **Table 1**.

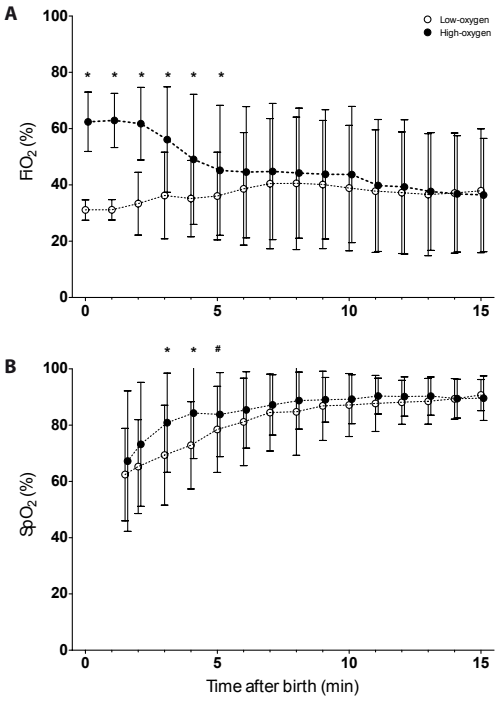

Figure 3: FiO_2 and SpO_2 during the first 15 minutes of resuscitation. A: FiO_2 was significantly different between the groups during the first 5 minutes of resuscitation (* $p < 0.01$). B: SpO_2 was significantly higher in the high-oxygen group at 3, 4 and 5 minutes of life (* $p < 0.01$, # $p < 0.05$).

Resuscitation

In 188 (97%) cases, preterm infants required respiratory support directly after birth. The FiO_2 was significantly higher in the high-oxygen group during the first five minutes of resuscitation (**Figure 3**A).

The FiO_2 in the low-oxygen group was increased to a mean of 40% (interquartile range 30-42%) by seven minutes after birth, while FiO_2 in the high-oxygen group was lowered to a mean of 40% (interquartile range 21-53%) by 11 minutes after birth. In both groups, approximately 26% of the infants received room air at transports to the NICU (**Table 2**).

Table 2: Resuscitation parameters.

	Low-oxygen initial FiO_2 30% n=99	**High-oxygen** initial FiO_2 65% n=94
Apgar score at 5 min	8 (7 - 9)	9 (8 - 9)
Time to SpO_2 88% (m:ss)	5:45 (3:49 - 7:51)	3:14 (2:08 - 5:24)*
Oxygen load first 15 min (ml/kg)#	1103 (1026 - 1453)	1497 (1178 - 2069)*
Intubation in delivery room	31 (31%)	28 (30%)
Receiving room air at transfer to NICU	24 (24%)	27 (28%)
Time to transfer to NICU (min)	20 (17 - 28)	21 (16 - 27)

Data are presented as median (interquartile range) or n (%). * $p < 0.001$. # Assuming equal exhaled tidal volume of 4 ml/kg and respiratory rate 60/min. SpO_2 = oxygen saturation measured by pulse oximetry, NICU = neonatal intensive care unit, FiO_2 = fraction of inspired oxygen

At three, four, and five minutes, the SpO_2 was higher in the high-oxygen group ($p<0.05$, **Figure 3**B) and subsequently the time to achieve a SpO_2 of 88% was shorter in the high-oxygen group ($p = 0.01$). Other general resuscitation parameters, such as Apgar scores, were not significantly different between the groups (**Table 2**).

Clinical outcome

Neither the incidence of BPD nor survival without BPD were significantly different between the groups (**Table 3**). Mortality tended to be higher in the high-oxygen group, but did not reach statistical significance. Also in the per protocol analysis, the incidence of BPD (p=0.162), mortality (p=0.221), and survival without BPD (p=0.771) were not different between the groups. In a subgroup analysis with infants with GA ≤ 28 weeks, no differences were found in the incidence of BPD (p=0.495), mortality (p=0.387), or survival without BPD (p=0.646) between an initial FiO_2 of 30% or 65%.

Table 3: Clinical outcome parameters.

		Low-oxygen initial FiO_2 30% n=99	**High-oxygen** initial FiO_2 65% n=94
BPD		23 (24%)	14 (17%)
Mortality		6 (6%)	10 (11%)
Survival without BPD		71 (72%)	70 (75%)
Duration of oxygen treatment (d)		2 (0 - 31)	2 (0 - 23)
Duration of mechanical ventilation (d)		2 (0 - 12)	1 (0 - 9)
Surfactant treatment		45 (45%)	41 (43%)
PDA	**Pharmacologically treated**	28 (28%)	23 (24%)
	Surgically treated	7 (7.1%)	5 (5.3%)
NEC ≥ grade 2		4 (4.0%)	3 (3.2%)
ROP ≥ grade 2		6 (6.1%)	5 (5.3%)
IVH ≥ grade 2		8 (8.1%)	10 (10.5%)

Data are presented as median (interquartile range) or n (%). # BPD was diagnosed according to the physiological criteria described by Walsh et al.[13]. BPD = bronchopulmonary dysplasia, PDA = patent ductus arteriosus, NEC = necrotising enterocolitis, ROP = retinopathy of prematurity, IVH = intraventricular haemorrhage.

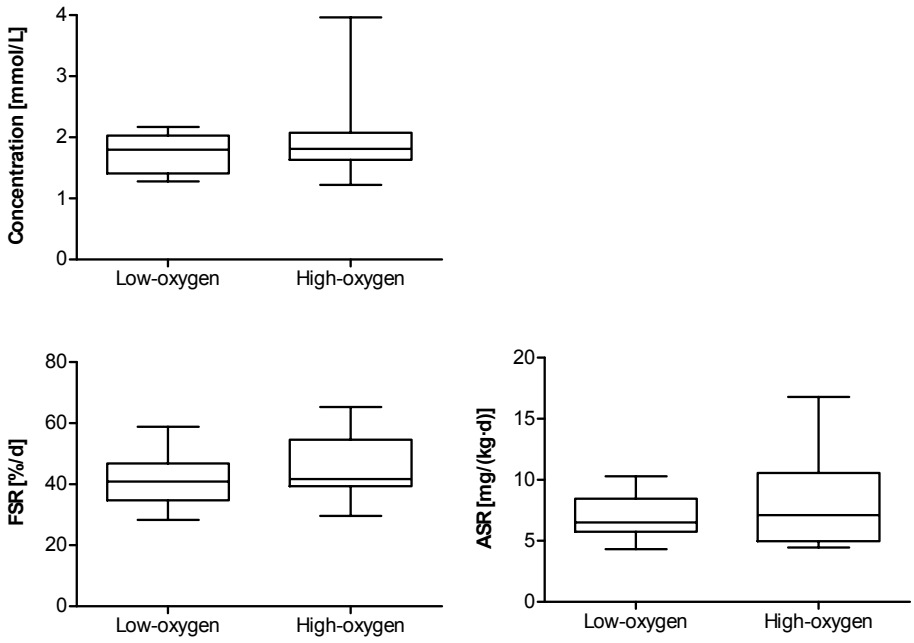

Figure 4: Glutathione concentration and synthesis rates on day 2.
Glutathione concentration, fractional synthesis rate (FSR) and absolute synthesis rate (ASR) were not different between the low-oxygen group (n=15) and the high-oxygen group (n=13).

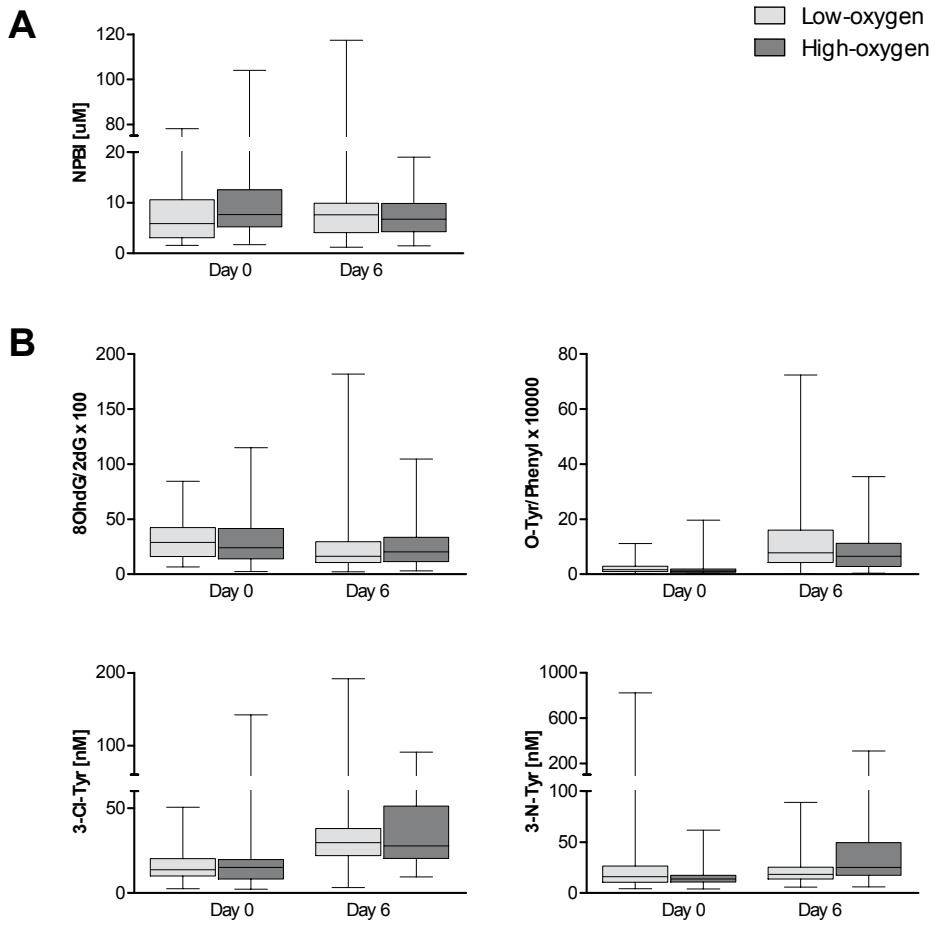

Figure 5: Oxidative stress markers on day 0 and day 6.
A: Plasma non protein bound iron (NPBI) was not different between groups (day 0: n=28 and 19, day 6: n=22 and 19 in low-oxygen group and high oxygen group respectively). B: Urinary oxidative stress markers were not different between the groups (day 0: n=41 and 34, day 6: n=46 and 34 in low-oxygen group and high-oxygen group respectively).

Oxidative stress

GSH concentration and synthesis were not significantly different between the groups (**Figure 4**). Also, NPBI and urinary oxidative stress markers were not significantly different on the first day or on the 6th day of life (**Figure 5**).

DISCUSSION

For years, there has been a discussion on the optimal dose of oxygen for the resuscitation of preterm infants after birth, because both hyperoxia and hypoxia can have detrimental effects. This study demonstrates that resuscitation with an initial FiO_2 of 30% is safe, but does not result in either increased survival without BPD or decreased oxidative stress compared to a FiO_2 of 65%. The more immature preterm infants are more likely to need supplemental oxygen due to immature lungs[6], but might also be more susceptible to oxidative damage. Therefore, we performed a subgroup analysis of preterm infants with GA ≤ 28 weeks demonstrating no differences in the incidence of BPD or survival without BPD.

At present, this study is the largest study examining the optimal oxygen concentration for the resuscitation of preterm infants. Our results add to the study of Vento et al. that showed that resuscitation with an initial FiO_2 of 30% decreased oxidative stress and lowered the incidence of BPD compared to an FiO_2 of 90% in infants < 28 weeks of gestation[11]. In that study, the FiO_2 needed individual adjustment in both groups to a mean FiO_2 of 55% by five minutes of life. Also in our study, FiO_2 was adjusted in both groups based on individual SpO_2 recordings. This resulted in a mean FiO_2 around 40% after six minutes in both groups, which remained relatively constant during the entire resuscitation. Difference between the two studies in the adjusted oxygen dose cannot be explained by different target SpO_2, as our targets were higher. More likely, the higher gestational age, and thus further developed lungs, explains the difference. Combining these data, it can be concluded that the optimal dose lies between 30% and 65%, but should not be as high as 90% and individual monitoring and adjustments are needed.

The amount of oxygen administered was significantly different during the first 5 minutes. Because newborn's lungs need some time to become aerated, the oxygen dose that actually has entered the patient is unknown. Unfortunately, we were not able to measure tidal volume or respiratory rate. Assuming equal respiratory rate and tidal volumes, the oxygen load was significantly higher in the high-oxygen group than in the low-oxygen group resulting in increased SpO_2 levels and reduced time to achieve a SpO_2 of 88%. Although we could not demonstrate differences in short-term clinical outcome or markers of oxidative stress, this still may have subtle effects on the long-term outcome.

We acknowledge the following limitations of our study. First, this study was not powered to detect differences in sole mortality. In developed countries, mortality is relatively low in preterm infants with a GA <32 weeks, with reported mortality rates varying between 10-13%[19 20]. It can be argued that there is a trend towards higher mortality in the high-oxygen group. A meta-analysis of available data could be helpful to determine the effect on mortality. Second, the generalizability of our results might be threatened by selection of cases in which time was available to obtain antenatal informed consent. In a recent publication by Rich et al., it was shown that eligible but non-enrolled infants had different demographics, e.g. less exposure to antenatal steroids, and worse clinical outcomes than enrolled infants[21]. In our study, all enrolled

infants received at least one dose of antenatal steroids. Prenatal steroids are indicated for the prevention of respiratory distress, and are associated with increased antioxidant enzyme activity, which reduces susceptibility to hypoxia and to oxidative damage as a result of hyperoxia[22]. Therefore, the studied cases may have shown less morbidity, such as less BPD, than the general population of very preterm infants. On the other hand, the majority of included infants were born prematurely because of pre-eclampsia (n=73) and chorioamnionitis (n=23), which are associated with increased oxidative stress[23 24]. The oxidative burden in these infants might already be so high that the differences in oxygen supplementation between our groups may not have had great influence on the oxidative burden. Taken together, future studies should use a waiver of consent in order to include preterm infants after imminent delivery.

The main strengths of our study are the high number of included infants, the fact that we studied the initial FiO_2 in a double-blind trial, and that we included very preterm infants in a trial with an acute intervention. The blinded setting warranted that FiO_2 adjustments during resuscitation were not influenced by the knowledge of the initial FiO_2. Instead, adjustments in the FiO_2 were based solely on the infants' condition. Therefore, our results resemble the situation in clinical practice and allow generalising of our results to clinical practice. Moreover, we studied well-known and reliable biomarkers of oxidative stress, nitrosative stress, and inflammation due to free radicals which positively backed our clinical results.

Adequate ventilation is important for oxygen supplementation. Without appropriate aeration of the lung (i.e., establishing functional residual capacity), oxygen supplementation will not warrant effect and may result in overexposure of the lung to oxygen[6]. Preterm infants are, however, difficult to ventilate in the immediate postnatal phase due to immaturity of the lung. Furthermore, preterm infants lack sufficient pulmonary antioxidant defences to overcome the sudden exposure to oxygen[25 26], which makes them more susceptible for oxidative lung damage. Therefore, it seems preferable to initiate oxygen supplementation after birth with the lowest oxygen concentration that is safe.

In this study, we demonstrated that resuscitation of preterm infants with 30% oxygen is as safe as resuscitation with 65% oxygen, but does not offer benefits with regard to survival without BPD. However, the amount of oxygen administered to the infants in the low-oxygen group was significantly lower for several minutes. Although this did not result in differences in short-term clinical outcome or oxidative stress, the long-term follow-up of these infants will unravel if there were subtle differences that were not demonstrated in the neonatal period. Based on the present data, we advise to start the resuscitation of preterm infants with GA <32 weeks with an initial FiO_2 of 30%. FiO_2 should be subsequently adjusted based on SpO_2 measurements according to the approach proposed by Dawson et al.[6].

REFERENCES

1. Lee AC, Cousens S, Wall SN, Niermeyer S, Darmstadt GL, Carlo WA, et al. Neonatal resuscitation and immediate newborn assessment and stimulation for the prevention of neonatal deaths: a systematic review, meta-analysis and Delphi estimation of mortality effect. *BMC Public Health* 2011;11 Suppl 3: S12.
2. Vento M, Asensi M, Sastre J, Garcia-Sala F, Pallardo FV, Vina J. Resuscitation with room air instead of 100% oxygen prevents oxidative stress in moderately asphyxiated term neonates. *Pediatrics* 2001; 107(4):642-7.
3. Saugstad OD, Ramji S, Soll RF, Vento M. Resuscitation of Newborn Infants with 21% or 100% Oxygen: An Updated Systematic Review and Meta-Analysis. *Neonatology* 2008;94(3):176-82.
4. O'Donnell CP. 'Resuscitation' of extremely preterm and/or low-birth-weight infants - time to 'call it'? *Neonatology* 2008;93(4):295-301.
5. Dawson JA, Kamlin CO, Vento M, Wong C, Cole TJ, Donath SM, et al. Defining the Reference Range for Oxygen Saturation for Infants After Birth. *Pediatrics* 2010;125(6):e1340-7.
6. Dawson JA, Vento M, Finer NN, Rich W, Saugstad OD, Morley CJ, et al. Managing Oxygen Therapy during Delivery Room Stabilization of Preterm Infants. *J Pediatr* 2011;160(1):158-61.
7. Vento M, Escobar J, Cernada M, Escrig R, Aguar M. The use and misuse of oxygen during the neonatal period. *Clinics in perinatology* 2012;39(1):165-76.
8. Perlman JM, Wyllie J, Kattwinkel J, Atkins DL, Chameides L, Goldsmith JP, et al. Part 11: neonatal resuscitation: 2010 International Consensus on Cardiopulmonary Resuscitation and Emergency Cardiovascular Care Science With Treatment Recommendations. *Circulation* 2010;122(16 Suppl 2):S516-38.
9. Wang CL, Anderson C, Leone TA, Rich W, Govindaswami B, Finer NN. Resuscitation of preterm neonates by using room air or 100% oxygen. *Pediatrics* 2008;121(6):1083-9.
10. Escrig R, Arruza L, Izquierdo I, Villar G, Saenz P, Gimeno A, et al. Achievement of targeted saturation values in extremely low gestational age neonates resuscitated with low or high oxygen concentrations: a prospective, randomized trial. *Pediatrics* 2008;121(5):875-81.
11. Vento M, Moro M, Escrig R, Arruza L, Villar G, Izquierdo I, et al. Preterm Resuscitation With Low Oxygen Causes Less Oxidative Stress, Inflammation, and Chronic Lung Disease. *Pediatrics* 2009;124(3):e439-49.
12. Rook D, Schierbeek H, van der Eijk A, Longini M, Buonocore G, Vento M, et al. Resuscitation of very preterm infants with 30% vs. 65% oxygen at birth: study protocol for a randomized controlled trial. *Trials* 2012;13(1):65.
13. Walsh MC, Yao Q, Gettner P, Hale E, Collins M, Hensman A, et al. Impact of a physiologic definition on bronchopulmonary dysplasia rates. *Pediatrics* 2004;114(5):1305-11.
14. Paffetti P, Perrone S, Longini M, Ferrari A, Tanganelli D, Marzocchi B, et al. Non-protein-bound iron detection in small samples of biological fluids and tissues. *Biol Trace Elem Res* 2006;112(3):221-32.
15. Solberg R, Andresen JH, Escrig R, Vento M, Saugstad OD. Resuscitation of hypoxic newborn piglets with oxygen induces a dose-dependent increase in markers of oxidation. *Pediatr Res* 2007;62(5):559-63.
16. Orhan H, Vermeulen NP, Tump C, Zappey H, Meerman JH. Simultaneous determination of tyrosine, phenylalanine and deoxyguanosine oxidation products by liquid chromatography-tandem mass spectrometry as non-invasive biomarkers for oxidative damage. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004;799(2):245-54.
17. Ehrenkranz RA, Walsh MC, Vohr BR, Jobe AH, Wright LL, Fanaroff AA, et al. Validation of the National Institutes of Health consensus definition of bronchopulmonary dysplasia. *Pediatrics* 2005;116(6): 1353-60.
18. The CRIB (clinical risk index for babies) score: a tool for assessing initial neonatal risk and comparing performance of neonatal intensive care units. The International Neonatal Network. *Lancet* 1993; 342(8865):193-8.

19. Guerrot AM, Chadie A, Torre S, Rondeau S, Pinto Cardoso G, Abily-Donval L, et al. Compared outcomes of very preterm infants born in 2000 and 2005. *Acta Paediatr* 2012.
20. Horbar JD, Carpenter JH, Badger GJ, Kenny MJ, Soll RF, Morrow KA, et al. Mortality and neonatal morbidity among infants 501 to 1500 grams from 2000 to 2009. *Pediatrics* 2012;129(6):1019-26.
21. Rich W, Finer NN, Gantz MG, Newman NS, Hensman AM, Hale EC, et al. Enrollment of extremely low birth weight infants in a clinical research study may not be representative. *Pediatrics* 2012;129(3): 480-4.
22. Vento M, Aguar M, Escobar J, Arduini A, Escrig R, Brugada M, et al. Antenatal steroids and antioxidant enzyme activity in preterm infants: influence of gender and timing. *Antioxidants & redox signaling* 2009;11(12):2945-55.
23. Raijmakers MT, Roes EM, Poston L, Steegers EA, Peters WH. The transient increase of oxidative stress during normal pregnancy is higher and persists after delivery in women with pre-eclampsia. *Eur J Obstet Gynecol Reprod Biol* 2008;138(1):39-44.
24. Cheah FC, Pillow JJ, Kramer BW, Polglase GR, Nitsos I, Newnham JP, et al. Airway inflammatory cell responses to intra-amniotic lipopolysaccharide in a sheep model of chorioamnionitis. *Am J Physiol Lung Cell Mol Physiol* 2009;296(3):L384-93.
25. Frank L, Sosenko IR. Prenatal development of lung antioxidant enzymes in four species. *J Pediatr* 1987; 110(1):106-10.
26. Frank L, Sosenko IR. Failure of premature rabbits to increase antioxidant enzymes during hyperoxic exposure: increased susceptibility to pulmonary oxygen toxicity compared with term rabbits. *Pediatr Res* 1991;29(3):292-6.

Chapter 5

Resuscitation of preterm infants with 30% or 65% oxygen at birth: neurodevelopmental outcome at 2 years of age

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ABSTRACT

Introduction

Both hypoxia and hyperoxia during resuscitation can have detrimental effects on preterm infants. Previously, we demonstrated in a double-blind, randomized trial that initial supplementation with 30% oxygen during resuscitation of preterm infants after birth is as safe as 65% with regard to oxidative stress and short-term clinical outcome. The long-term follow-up of these infants might unravel if there were subtle differences that did not manifest in the neonatal period. The objective of this study was to compare neurodevelopmental outcome at 2 years of age after resuscitation of preterm infants with an initial FiO_2 of 30% or 65%.

Methods

In a double-blind, randomized trial, preterm infants with a gestational age (GA) <32 weeks were randomized to start resuscitation after birth with either 30% (low-oxygen group) or 65% oxygen (high-oxygen group). Initial FiO_2 was blinded for the resuscitation team and was adjusted based on oxygen saturation measured by pulse oximetry (SpO_2). Neurodevelopmental outcome was assessed at 2 years of age using the third edition of the Bayley Scales of Infant and Toddler Development (BSID-III), which was performed by certified neurodevelopmental psychologists who were blinded for initial randomization.

Results

In total, 193 infants were included with a mean GA of 28⅘ ± 2⅐ weeks and a mean birth weight 1076 ± 347 gram (not different between groups). Currently, follow-up of 126 infants is completed with a follow-up rate of 85%. Of the infants in whom follow-up was complete, 86% was alive without severe neurodevelopmental impairment at two years of age. Mean Mental Development Index (MDI) was 98 ± 11 and mean Psychomotor Development Index (PDI) was 102 ± 13.

Conclusions

De-blinding will be performed after follow-up is completed for all infants (May 2014).

INTRODUCTION

During resuscitation, the majority of preterm infants need active respiratory support with, in most cases, administration of additional oxygen[1]. Resuscitation of infants at birth with 100% oxygen is, however, known to increase oxidative stress with concomitant deleterious effects[2 3]. In preterm infants, increased oxidative stress is associated with serious neonatal diseases like bronchopulmonary dysplasia, retinopathy of prematurity and periventricular leukomalacia[4]. Therefore, reduction of the oxygen load during resuscitation, especially for preterm infants, is necessary.

Several small studies investigated the optimal FiO_2 for resuscitating preterm infants by comparing a low and high initial FiO_2[5-7]. From these studies, it can be concluded that initiating the resuscitation of preterm infants with room air is too low, while starting with 90% FiO_2 seems to be too high. To assess the safety and efficacy of starting resuscitation of preterm infants with low oxygen concentrations, we compared 30% oxygen with an intermediate FiO_2 in a double-blind, randomized study. In **Chapter 4**, we demonstrated that resuscitation of preterm infants with an initial FiO_2 of 30% was as safe as an initial FiO_2 of 65%, but did not result in either increased survival without BPD or decreased oxidative stress compared to 65%. However, the administered FiO_2 and subsequently the oxygen saturation measured by pulse oximetry (SpO_2) were significantly higher in the group starting with an initial FiO_2 of 65%.

Although we could not demonstrate differences in short-term clinical outcome or markers of oxidative stress, this difference in oxygen load might have effects on long-term outcome. Therefore, we performed long-term follow-up and hypothesized that resuscitation of preterm infants with an initial FiO_2 of 30% results in improved neurodevelopmental outcome at 2 years of age compared to 65% oxygen.

SUBJECTS AND METHODS

This double-blind, randomized trial was performed in the Erasmus MC - Sophia Children's Hospital (Rotterdam, the Netherlands) between August 2008 and January 2012. The study was investigator-initiated without external funding. Study protocol was approved by the Erasmus MC Medical Ethics Committee.

Subjects

Preterm infants with a gestational age (GA) below 32 weeks born at the Erasmus MC - Sophia Children's Hospital were eligible for this study. Assessment of GA was based on early foetal ultrasonography or on the date of last menstrual period. Written informed consent was obtained from all parents prenatally, since the intervention was performed directly after birth. Mothers admitted to Erasmus MC - Sophia Children's Hospital at risk for preterm delivery before 32 weeks

of gestation were approached for participation in the study. Exclusion criteria included major congenital malformations or chromosome defects identified prior to birth. Infants with major congenital malformations, chromosome defects, and metabolic, endocrine or renal disorders identified after randomization were also excluded from analysis.

Intervention

Study design and resuscitation procedures have been described previously[8]. In short, preterm infants were randomized to start resuscitation with either 30% oxygen (low-oxygen group) or 65% oxygen (high-oxygen group) for which the physician was blinded. The objective of the resuscitation was to achieve a target SpO_2 of 88-94% at 10 minutes after birth. If the pulse rate remained stable and over 100 beats per minute, no intervention was advised. At all times, the physician could adjust the FiO_2 if the clinical situation was not satisfactory (e.g. persistent bradycardia or SpO_2 >94%). The FiO_2 could then be manually adjusted to the desired FiO_2, without knowledge of the initial FiO_2 to which the infant was randomized.

Neurodevelopmental outcome

As part of standard care, all preterm infants born with a GA <32 weeks born at the Erasmus MC - Sophia Children's Hospital are assessed at the corrected age of two years.

Follow-up consists of a complete physical examination and testing of mental and psychomotor development with the third edition of the Bayley Scales of Infant and Toddler Development (BSID-III)[9]. Scores are reported as Mental Development Index (MDI) and Psychomotor Development Index (PDI), standardized with a mean score of 100 and a standard deviation of 15 points. PDI is reported as composite score, with motor subscales for fine motor and gross motor functions. Children in whom cerebral palsy (CP) is suspected are also evaluated by a child neurologist. Language skills are tested using a lexicon for the Dutch language list to assess the infants' vocabulary[10]. The neurodevelopmental psychologists are unaware of inclusion in the resuscitation study and are therefore blinded for the initial randomization.

Definitions

Cerebral palsy (CP) was classified by the Gross Motor Function Classification System (GFMCS)[11]. Mild neurodevelopmental impairment at follow-up was defined as at least one of the following outcomes: CP grade 1 and a MDI and/or PDI of 70–84 points. Severe neurodevelopmental impairment was defined as at least one of the following: CP grade 2–5, MDI and/or PDI <70, bilateral blindness or bilateral hearing loss requiring amplification.

Statistics

Primary outcome for this study was survival without neurodevelopmental impairment at the corrected age of two years. Power calculation in the original study was based on the incidence of BPD and showed that, with an incidence of 30% and an expected reduction of 15% using

1-sided test, 100 infants per group were needed to find a statistically significant reduction with an α of 0.05 and a power of 0.80. After de-blinding, differences between groups will be assessed using the Mann-Whitney test for continuous measurements and the Chi-square test for categorical measurements ($p < 0.05$). We will perform an adjusted analysis with correction for the major confounders; gestational age, birth weight, educational level of the mother and social economic status.

RESULTS

From a total of 781 eligible preterm infants, 193 were analyzed in the study (**Figure 1**). The main reason for missed inclusion was imminent preterm delivery, leaving no time to obtain informed consent prior to birth. Due to the acute character of the resuscitation of preterm infants, 67

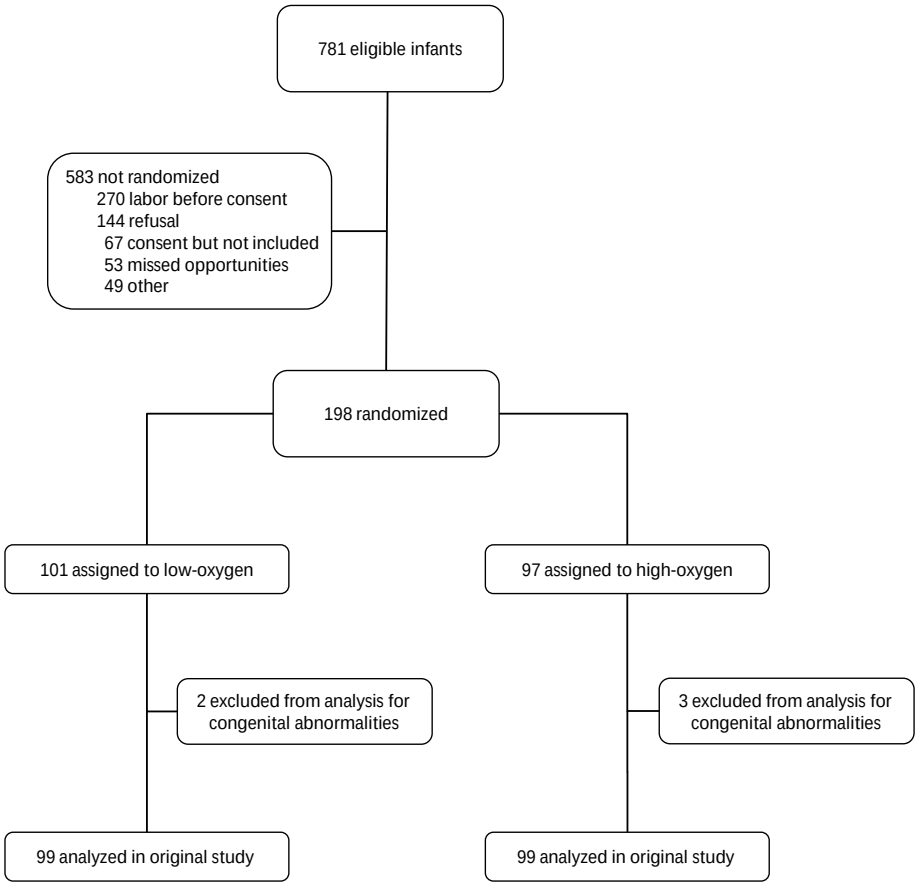

Figure 1: Trial profile.

Table 1: Clinical and obstetric characteristics.

	Low-oxygen	High-oxygen
N (male : female)	99 (44 : 55)	94 (43 : 51)
Gestational age (weeks)	28 ⅘ ± 2 ⅕	28 ⅘ ± 2 ⅖
Birth weight (g)	1056 ± 340	1096 ± 351
Birth weight Z-score	-1.0 ± 1.4	-0.9 ± 1.3
CRIB score#	3.6 ± 3.8	3.1 ± 3.4
Corticosteroids (%)	100%	100%
Incomplete (%)	7%	11%
Type of delivery (vaginal: CS)	31 : 68	33 : 61
Umbilical cord pH	7.29 ± 0.09	7.28 ± 0.09

Data are presented as mean ± SD or as n (%). # The CRIB score (Clinical Risk Index for Babies) indicates the degree of illness[12]. The score has a maximum of 23 points and is positively correlated with the severity of illness. CS = caesarean section.

infants were missed despite consent. General demographic and obstetric characteristics are depicted in **Table 1**.

Currently, follow-up is completed for the first 126 included infants of whom 10 died during the neonatal period. Of the 116 survivors, 17 could not be located or refused to participate (follow up rate of 85%). Additionally, due to lack of cooperation of the child during the test (n=14), severe cerebral palsy (n=2) or large difference between fine and gross motor function (n=5), the composite motor score was missing. Neurodevelopmental outcome at two years of corrected age for the total group is depicted in **Table 2**. Of the infants in whom follow-up

Table 2: Neurodevelopmental outcome at two years of corrected age.

	Mean ± SD or n (%)	Mildly impaired n (%)	Severely impaired n (%)
MDI (n=99)	98 ± 11	6 (6.1%)	2 (2.0%)
PDI (n=78)	102 ± 13	1 (1.3%)	3 (3.8%)
Fine motor subscale (n=85)	11.7 ± 2.7	0	1 (1.2%)
Gross motor subscale (n=74)	8.8 ± 2.8	5 (6.7%)	3 (4.1%)
Language score (n=96)	92 ± 16	n/a	n/a
CP	4 (4.0%)	2 (2.0%)	2 (2.0%)
Bilateral blindness	0	n/a	n/a
Bilateral hearing loss requiring amplification	0	n/a	n/a
Neurodevelopmental impairment	12 (12.1%)	7 (7.1%)	5 (5.0%)

MDI = Mental Development Index, PDI = Psychomotor Development Index, n/a = not applicable.
Mild impairment was defined as a MDI and/or PDI of 70–84 points, or cerebral palsy (CP) grade 1. Severe impairment was defined as a MDI and/or PDI of < 70 points, a subscale score <4 points, CP grade 2–5, bilateral blindness or bilateral hearing loss requiring amplification.

was complete, 86% was alive without severe neurodevelopmental impairment at two years of age.

DISCUSSION

Previously, we demonstrated that short-term clinical outcome and oxidative stress were similar between an initial FiO_2 of 30% and 65% oxygen during resuscitation of preterm infants (Chapter 4). Administered oxygen and subsequently the SpO_2 were significantly higher during the first minutes upon administration of 65% oxygen. Although we could not demonstrate differences in short-term clinical outcome or markers of oxidative stress, the difference in oxygen load might have effects resulting in different long-term outcome. We hypothesized that resuscitation of preterm infants with an initial FiO_2 of 30% results in improved neurodevelopmental outcome at 2 years of age compared to 65% oxygen. Currently (December 2012), follow up is completed for the first 126 included infants, with a follow up rate of 85%. Of these 126 infants, 86% was alive without major handicaps at two years of age with a mean MDI of 98 ± 11 and PDI of 102 ± 13. De-blinding will be performed after follow-up is completed of all infants (May 2014).

REFERENCES

1. O'Donnell CP. 'Resuscitation' of extremely preterm and/or low-birth-weight infants - time to 'call it'? *Neonatology* 2008;93(4):295-301.
2. Vento M, Asensi M, Sastre J, Garcia-Sala F, Pallardo FV, Vina J. Resuscitation with room air instead of 100% oxygen prevents oxidative stress in moderately asphyxiated term neonates. *Pediatrics* 2001; 107(4):642-7.
3. Saugstad OD, Ramji S, Soll RF, Vento M. Resuscitation of Newborn Infants with 21% or 100% Oxygen: An Updated Systematic Review and Meta-Analysis. *Neonatology* 2008;94(3):176-82.
4. Saugstad OD. Update on oxygen radical disease in neonatology. *Curr Opin Obstet Gynecol* 2001;13(2): 147-53.
5. Wang CL, Anderson C, Leone TA, Rich W, Govindaswami B, Finer NN. Resuscitation of preterm neonates by using room air or 100% oxygen. *Pediatrics* 2008;121(6):1083-9.
6. Escrig R, Arruza L, Izquierdo I, Villar G, Saenz P, Gimeno A, et al. Achievement of targeted saturation values in extremely low gestational age neonates resuscitated with low or high oxygen concentrations: a prospective, randomized trial. *Pediatrics* 2008;121(5):875-81.
7. Vento M, Moro M, Escrig R, Arruza L, Villar G, Izquierdo I, et al. Preterm Resuscitation With Low Oxygen Causes Less Oxidative Stress, Inflammation, and Chronic Lung Disease. *Pediatrics* 2009;124(3):e439-49.
8. Rook D, Schierbeek H, van der Eijk A, Longini M, Buonocore G, Vento M, et al. Resuscitation of very preterm infants with 30% vs. 65% oxygen at birth: study protocol for a randomized controlled trial. *Trials* 2012;13(1):65.
9. Bayley N. *Bayley Scales of Infant and Toddler Development, Third Edition*. San Antonio: Pearson assessment & information, 2005.
10. Schlichting JEPT, Lutje Spelberg HC. *Lexilijst Nederlands*. Lisse: Swets Test Publishers, 2002.
11. Palisano R, Rosenbaum P, Walter S, Russell D, Wood E, Galuppi B. Development and reliability of a system to classify gross motor function in children with cerebral palsy. *Developmental medicine and child neurology* 1997;39(4):214-23.
12. The CRIB (clinical risk index for babies) score: a tool for assessing initial neonatal risk and comparing performance of neonatal intensive care units. The International Neonatal Network. *Lancet* 1993; 342(8865):193-8.

Chapter 6

Observing the resuscitation of very preterm infants: Are we able to follow the oxygen saturation targets?

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ABSTRACT

Introduction

Since 2010, the European Resuscitation Council (ERC) guidelines advise oxygen saturation (SpO_2) targets for the first 10 minutes of resuscitation after birth. Unfortunately, the control of SpO_2 in newborn infants is difficult. The objective of this study was to determine to what extent SpO_2 levels match the ERC targets during the resuscitation of very preterm infants, and how well the SpO_2 is kept within the high and low limits until the infants are transported to the NICU.

Methods

In a single-centre observational study, the SpO_2 and fraction of inspired oxygen (FiO_2) were collected during the resuscitation of very preterm infants with a gestational age (GA) ≤30 weeks.

Results

A total of 78 infants were included [median (IQR): GA 27⅐ (26-28⅐) weeks, birth weight 945 (780-1140) g]. During the initial 10 minutes after birth, large variations in SpO_2 were observed with deviations above the target [median (IQR)] of 4.4 (1.4-6.5) % SpO_2, and below the target of 8.2 (2.8-16.0) % SpO_2. After the first 10 minutes, the SpO_2 levels were respectively above and below the limit for 11 (0-27) % and 8 (0-23) % of the time.

Conclusions

During the resuscitation of very preterm infants, large deviations of the SpO_2 from the ERC targets are observed. During the first minutes of resuscitation the deviations were likely caused by an inability to control the SpO_2, whereas later deviations were due to weaning, pauses in respiratory support (i.e. intubation) and over exposure to oxygen. Changing the SpO_2 targets to a target range that depicts the acceptable deviation might be helpful in providing better respiratory support.

INTRODUCTION

During resuscitation of preterm infants, supplemental oxygen therapy is often used to reach and maintain adequate oxygenation. Adequate oxygenation is essential in preterm infants because both hypoxia and hyperoxia can have detrimental effects on the organs, and even fluctuations in oxygenation can be damaging[1-3]. The damage to the organs is caused by the formation of excessive oxygen free radicals[4]. The compromised anti-oxidative capacity of preterm infants and the need for a certain level of oxidative stress to initiate the adaptation from intra to extra uterine life make the control of oxygenation a delicate balance[5].

To prevent negative outcomes due to under- or overexposure to oxygen in newborn infants, the European Resuscitation Council (ERC)[6], American Heart Association (AHA)[7] and Australian and New Zealand Resuscitation Council (ARC NZRC)[8] guidelines advise pulse oximetry oxygen saturation (SpO_2) targets for the first 10 minutes after birth. These targets are based on observational studies of healthy term and preterm infants not needing any intervention during their resuscitation[9]. To reach and maintain these SpO_2 targets, the fraction of inspired oxygen (FiO_2) is titrated manually according to the SpO_2 measurement. Unfortunately, none of the resuscitation guidelines specify how the FiO_2 should be titrated to make sure SpO_2 targets are reached.

Literature shows that manual control of the SpO_2 is difficult, reporting time spent outside the target range of approximately 50% in neonatal intensive care units (NICU)[10-13]. Although the status of the infants and the tasks of the physicians in NICUs differ from those during resuscitations immediately after birth, it is likely that during resuscitation it is difficult for clinicians to keep SpO_2 within the recommended target range. It is unknown to what extent the SpO_2 targets are achieved. Therefore, the aim of this study was to determine to what extent SpO_2 levels matched ERC targets during the resuscitation immediately after birth of very preterm infants.

SUBJECTS AND METHODS

An observational study was performed at the Erasmus Medical Center - Sophia Children's Hospital, Rotterdam, the Netherlands, a level-III-c NICU with 33 beds[14]. The medical ethics committee of the Erasmus Medical Center approved this study and decided that informed consent was not needed because no interventions were imposed and no personal data was processed. Because of the observational nature of this study, there was no possibility to determine a sample size.

Subjects

Patients born with a GA ≤30 weeks in the study centre were eligible for inclusion in this study. Congenital or chromosomal defects were exclusion criteria.

Local resuscitation protocol

The ERC guidelines were introduced 7 months prior to the start of this study. They were discussed amongst the staff prior to being adapted as the local resuscitation protocol, and are part of the education of resident physicians. A reminder of the SpO_2 targets was available in all resuscitation areas, together with a Dutch translation of the ERC 'Newborn life support algorithm'[15].

According to the local protocol, preterm infants were transferred to the resuscitation unit immediately after delivery, where at least 2 clinicians start to stabilize the infant. Resuscitation of infants <26 weeks GA is performed by a neonatologist or neonatal fellow. Measures were taken to prevent heat loss. Respiratory support was given, primarily with a T-piece resuscitator (Neopuff, Fisher & Paykel Healthcare, Auckland, New Zealand). A flow-inflating bag with pressure monitoring (Jackson Reese modification T-piece breathing system, Intersurgical, Wokingham, UK) was also available and could be used according to the physician's preferences. Contrary to the advice of the ERC guidelines to start all resuscitations with room air, local protocol advises to start resuscitation of infants with a GA ≤28 weeks with a FiO_2 of 0.30 (based on publications by Escrig et al., Vento et al. and Saugstad et al.[16-18]). Furthermore, FiO_2 should not be adjusted before an SpO_2 measurement is obtained, unless the heart rate, obtained from auscultation, drops below 100 beats per minute (bpm)[6]. A pulse oximeter sensor (Nellcor OxiMax Max-N, Covidien, Boulder, USA) was placed on the right hand or wrist to measure preductal SpO_2[19].

During the first 10 minutes after birth, the SpO_2 targets from the ERC guidelines were advised, i.e. 60%, 70%, 80%, 85%, and 90% at 2, 3, 4, 5, and 10 minutes after birth, respectively (**Figure 1A**). From the 10th minute onwards, the target range of the study centre's NICU was prescribed (85-93% SpO_2)[20]. When respiration was absent or insufficient, ventilation was initiated with sustained inflations, i.e. 5 inflations of 3 seconds, after which respiratory support could be optimized by adjusting the positive end expiratory pressure (PEEP) and/or peak inspiratory pressure (PIP) (initially set to 5 and 20 cmH_2O respectively). When respiration of the infants remained insufficient or if the infants remained hypoxic, endotracheal intubation was performed.

Outcome parameters

The primary outcome was the deviation of SpO_2 from a trend line drawn through the ERC targets and, after the 10th minute, the target range for SpO_2. Deviation from the target was assessed by the time spent above and below the target and by calculating the average absolute deviation per infant.

$$\textit{average absolute deviation} = \left(\frac{\Sigma_{\textit{measurements outside range}} |\text{target } SpO_2 - \text{measured } SpO_2|}{\textit{number of measurements outside range}} \right)$$

The deviation above the target was corrected for those moments when the SpO_2 was above the target while the infant was on room air (FiO_2 0.21). The secondary outcomes were the time to

obtain SpO_2 measurement, total resuscitation time, administered FiO_2, and number of intubation attempts.

Data collection

Measurements were obtained from the pulse oximeter (Nellcor N-600x, Covidien, Colorado, USA) and recorded with a frequency of 0.5 Hz from the first measurement until the infant was disconnected for transfer to the transport incubator. The FiO_2 was obtained (1 Hz) through an oxygen monitor (MX300, Teledyne Technologies, City of Industry, USA) that was connected to an oxygen sensor (M-15 STD, IT Dr. Gambert GmbH, Wismar, Germany) in the blender's bleed port (Bird Ultrablender, Cardinal Health, Dublin, USA). The time of birth was defined as the moment at which the APGAR timer was started. When the APGAR timer was not started, a time of 30 seconds prior to the infant being placed on the resuscitation unit was taken as the time of birth. In the 67 infants where the APGAR timer was started at birth it took a median (IQR) 30 (21-36) seconds for infants to be placed on the resuscitation unit. Data acquisition was performed on dedicated research computers, continuously running software specially written for this study (programmed in Labview 2011, National Instruments, Austin, USA).

RESULTS

Seventy-eight infants were included during an 8-month period (see **Table 1** for patient characteristics). The results are presented as median (IQR) unless stated otherwise. Of the 142 eligible infants, 42 were excluded [GA 27⅗ (25⅐-28⅘) weeks, birth weight 870 (763-1050) g] because they were included in one of two (interventional) studies that conflicted with the initial adjustment of the FiO_2[21]. The data of 21 patients [GA 27⅘ (26⅗-28⅘) weeks, birth weight 955 (828-1186) g] could not be used for analysis due to failure of the data acquisition. The failures of the data acquisition were purely technical; related to connections between the medical devices,

Table 1: Patient characteristics.

N (male : female)	41 : 37
GA (weeks)	27⅘ (26-28⅘)
GA ≤28 weeks (N)	51
Birth weight (g)	945 (780-1140)
Type of delivery (vaginal: CS)	33 : 45
Reason for preterm delivery (maternal : fetal)	11 : 31
Received full course of corticosteroids (N)	43
Umbilical cord pH	7.31 (7.05-7.48)
Apgar score at 5 min	8 (7-9)

Data represented as number (N) or median (IQR). CS = caesarean section, GA = gestational age.

the software, or the computer failing, which resulted in the data not being recorded. One infant was retrospectively excluded due to a congenital defect. There was a failure to start the APGAR timer in 11 cases (14%).

Deviation from the SpO_2 targets

During the first 10 minutes after birth, the time spent above [44 (12-66) %] and below [51 (27-82) %] the intended SpO_2 target was similarly distributed (**Table 2**), with a median deviation from the target of 8.2% SpO_2 (2.8-16.0). After the first 10 minutes, until the infant left the resuscitation area, 32% (14-46) of the time was spent outside of the NICU limits. The measured SpO_2 is plotted together with the ERC targets for SpO_2 and the NICU limits in **Figure 1A**. **Figure 1** also shows the measured pulse rate (**Figure 1B**), administered FiO_2 (**Figure 1C**), and the number of infants that were on the resuscitation unit at that specific time after birth and contributed data (**Figure 1D**). There were 21 large drops in the SpO_2 (<60%), which were all the result of

Table 2: Primary results.

SpO_2 deviation during the first 10 minutes after birth	
Time above ERC target (%)	44 (12-66)
Time below ERC target (%)	51 (27-82)
Average deviation above ECR target (% SpO_2)	4.4 (1.4-6.5)
Average deviation below ECR target (% SpO_2)	8.2 (2.8-16.0)
Average deviation (% SpO_2)	7.8 (5.8-12.5)
SpO_2 deviation after the first 10 minutes after birth	
Time above NICU limit (%)	11 (0-27)
Time below NICU limit (%)	8 (0-23)
Time outside NICU limit (%)	32 (14-46)
Average deviation above NICU limit (% SpO_2)	1.7 (0.3-2.5)
Average deviation below NICU limit (% SpO_2)	2.0 (0.0-5.1)
Average deviation (% SpO_2)	2.6 (1.3-4.5)
SpO_2 deviation during the entire resuscitation	
Total time above target (%)	25 (11-40)
Total time below target (%)	26 (14-39)
Average deviation above target (% SpO_2)	3.5 (2.4-5.4)
Average deviation below target (% SpO_2)	7.3 (3.2-13.3)
Average deviation (% SpO_2)	6.6 (4.6-9.6)
FiO_2	
Number of adjustments (N)	7 (3-10)
Average FiO_2 (%)	33.5 (26.8-44.9)
Min FiO_2 (%)	21.0 (20.5-22.4)
Max FiO_2 (%)	59.0 (36.9-99.3)
FiO_2 at the end of resuscitation (%)	26.2 (22.2-33.0)

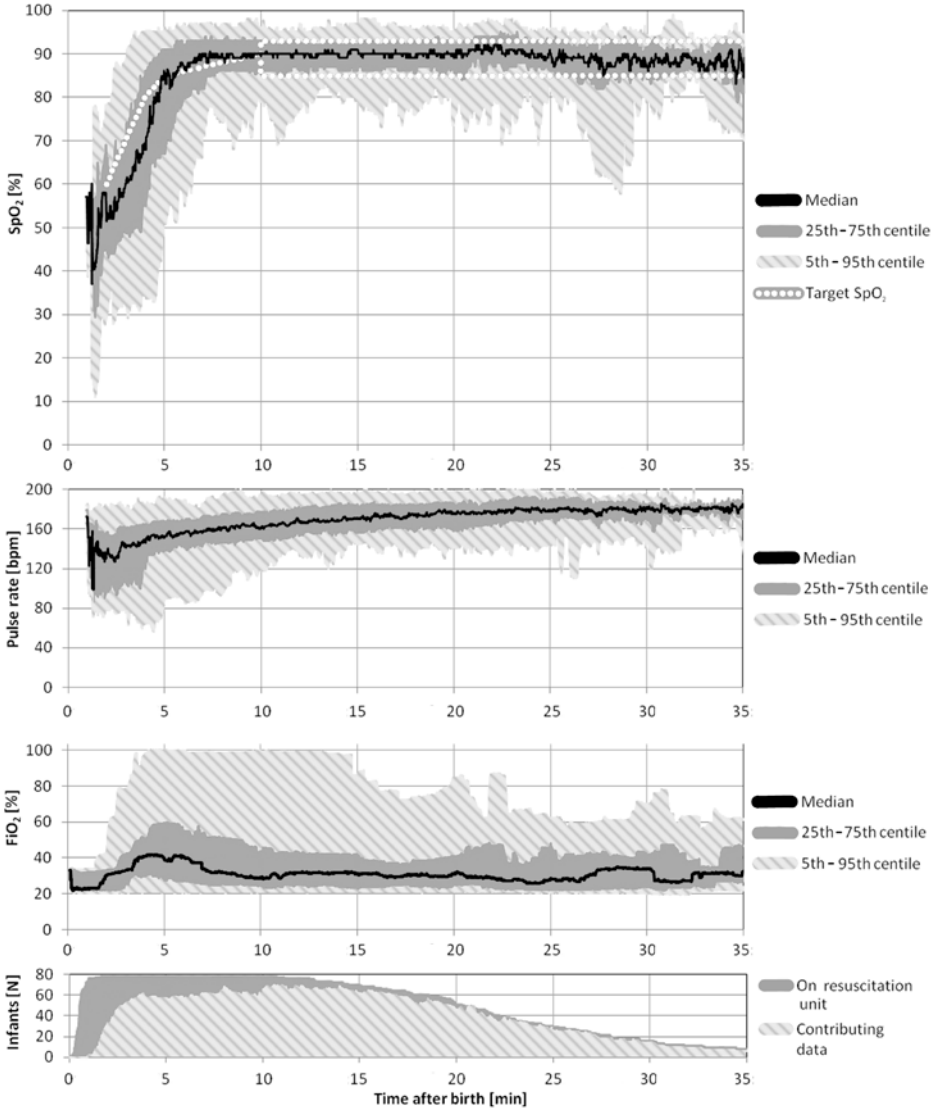

Figure 1: Measured oxygen saturation (SpO_2) and fraction of inspired oxygen (FiO_2) during the resuscitation of preterm infants (N=78).
A) Median, 5th, 25th, 75th and 95th percentile of the SpO_2 measured during resuscitation, plotted together with the European Resuscitation Council (ERC), and high and low SpO_2 targets, as used at the Erasmus Medical Center (Rotterdam, the Netherlands). B) Median, 5th, 25th, 75th and 95th percentile of the measured pulse rate. C) Median, 5th, 25th, 75th and 95th percentile of the FiO_2 administered during the resuscitation. D) Number of infants that were on the resuscitation unit at specific times after birth, and number of infants that were contributing to the data set.

intubation attempts. In 41 infants (53%), the SpO_2 was at some point above the target, while the FiO_2 was 0.21, for which the deviation was corrected. During the first 10 minutes after birth, this correction occurred for a total of 9% of the time (67 min) and for 3% of the time (64 min) during the remainder of the resuscitations. Only 6 infants (8%) remained inside the limits after the first 10 minutes.

Secondary outcomes

The infants spent 22:24 (19:08-28:01) minutes on the resuscitation unit. The interval between the moment that the infant was placed on the resuscitation unit and the first SpO_2 measurement was 1:29 (1:15-2:16) minutes. In 67 resuscitations (86%), the SpO_2 sensor was positioned on the extremity of the infant before the connector of the sensor was plugged into the monitor, which is the quickest method for obtaining an accurate measurement[22]. At the moment of the first ERC target (2 minutes after birth), the measurements from 33 infants were obtained (42%).

For infants with GA ≤28 weeks, the initial FiO_2 was 0.30 in 29 cases (57%), in one case it was set to 0.40, in the other cases room air was used. In one case the FiO_2 was corrected to 0.30 almost immediately. Two infants with a GA >28 weeks (8%) received an initial FiO_2 of 0.30. The FiO_2 was increased before there was an SpO_2 measurement in 16 cases. In 9 of these 16 cases (56%), we could confirm that it was because of a low heart rate. When leaving the resuscitation area, the median FiO_2 of all infants was 26.2% (22.2-33.0). When an infant needed to be intubated (N=28, 36%), 2 (1-2) attempts were needed to do so successfully. Thirty two infants (41%) left the resuscitation area with a nasal cannula, the others with mask ventilation (N=18, 23%).

DISCUSSION

This study determined to what extent SpO_2 levels matched the ERC targets during the resuscitation of preterm infants in daily practice. While the median of the observed infants followed the ERC targets quite nicely, it did deviate below the targets during the first 5 minutes after birth. Overall the variation in the SpO_2 was large. The average deviation from the targets was 6.6% SpO_2 (4.6-9.6), whereas the deviation more than doubled in the worst cases (95th centile 19.3% SpO_2) (**Figure 1A**).

There are several possible explanations for the large deviations during the first few minutes after birth. First, in some infants, the first SpO_2 measurement took longer to obtain (**Figure 2**), which is most likely caused by poor perfusion, or problems with sensor placement[23 24]. A longer time to obtain an SpO_2 measurement will increase the time until control over the SpO_2 is achieved, because the FiO_2 is not adjusted without an SpO_2 measurement unless the heart rate is below 100 bpm. Such a delay could cause a further deviation from the SpO_2 targets. Second, during the initial phase of resuscitation, ventilation of preterm infants is hampered by lung immaturity, resulting in inappropriate aeration of the lung, i.e. establishing functional

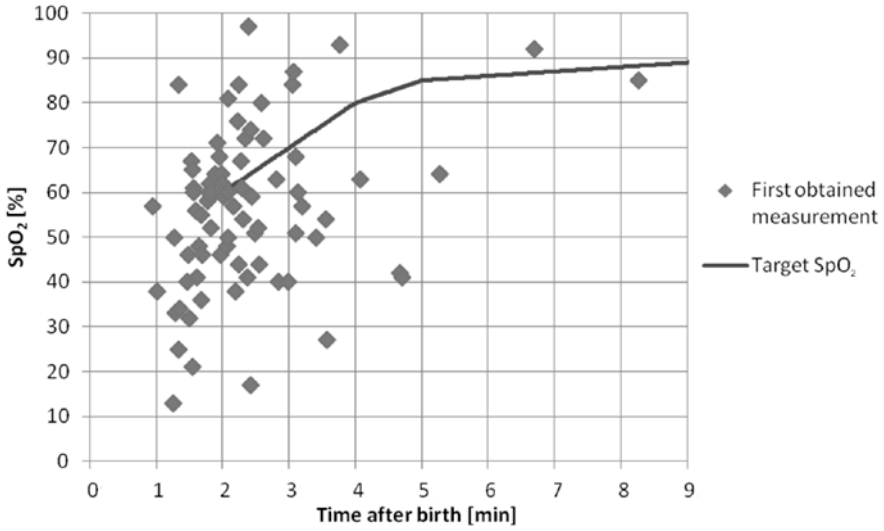

Figure 2: First oxygen saturation measurement (SpO_2) of the resuscitated infants (N=78) at the time after birth the SpO_2 measurement was obtained.

residual capacity[25]. Other explanations for suboptimal ventilation could be mask leaks or airway obstructions[26 27].

The median of the administered FiO_2 rose sharply on two occasions, before 2 and shortly after 3 minutes after birth. The first rise is likely due to the initial assessment of the infant, the second rise because the SpO_2 measurement became available. Between 4 and 5 minutes after birth the median of the SpO_2 rose to follow the targets more closely and a reduction of the variation in pulse rate was observed. These combined results of respiratory support, improved lung recruitment and perfusion, indicate that in most infants control adequate respiratory support was obtained at this point[27].

After the first 10 minutes, 32 (14-46) % of the time was spent outside the SpO_2 limits. On average 36 (31-47) % of the infants were outside the SpO_2 limits at any given time. Thus, even with adequate respiratory support, remaining between the high and low SpO_2 levels was challenging. Instability in the oxygenation was caused by, for example, a temporary halt in the respiratory support (i.e., tube placement or suctioning) but can also be caused by incomplete adaptation[28]. The time spent above the intended range was the result of the administration of a too high FiO_2 and could have been avoided by reducing the FiO_2. However, determining by how much the FiO_2 should be reduced is one of the major challenges in controlling the SpO_2, and the fear of low SpO_2 values might deter physicians from making rapid adjustments.

The APGAR time was not started in 14% of the resuscitations, indicating that the staff is not always fully focused on starting the timer. Not starting the timer will make the following of the SpO_2 targets more difficult, because an exact time after birth is not readily available to the

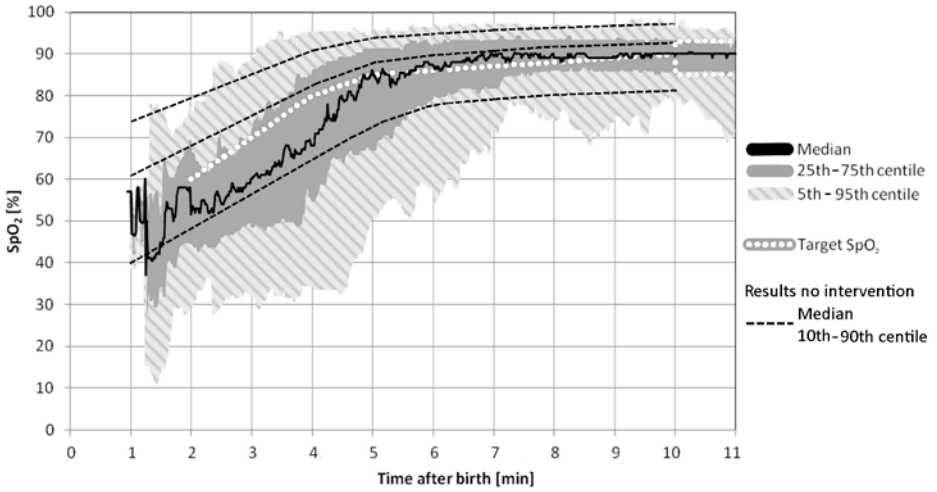

Figure 3: Comparison of the oxygen saturation (SpO_2) during resuscitation of preterm infants (N=78) to preterm infants (<32 weeks gestational age, N=32) that did not receive any medical intervention after birth as observed by Dawson et al.[9].
The number of infants on the resuscitation unit and the number that was contributing data can be found in Figure 1D.

physicians. Compliance with the local protocol to start resuscitation of infants ≤28 weeks GA with a FiO_2 of 30% was low (57%).

The ERC guidelines prescribe single value SpO_2 targets, while the AHA and ARC guidelines advise a narrow SpO_2 target range[7 8]. A target range provides physicians with information on what is considered to be an acceptable deviation, and could actually reduce the observed variation. A group of experts on the resuscitation of preterm infants has suggested using the 10th and 50th centile of the study of Dawson et al.[9] (the same study as which the current guidelines are based on), as the SpO_2 target range, which is a significantly lower low target than the other SpO_2 targets (**Figure 3**)[29].

When our results are compared to the observations of Dawson et al. of preterm infants (<32 weeks GA, N=39) who did not require medical intervention after birth, it seems likely that most infants were in a safe range with their SpO_2 values (**Figure 3**)[9]. However during the first 6 minutes after birth more than 25% of the observed infants had SpO_2 values that were below the 10th centile. Whether single value SpO_2 targets or target ranges result in more accurate control of the SpO_2 during routine clinical resuscitations needs to be determined. Furthermore, it remains unknown which SpO_2 targets or target range provide the best compromise between exposure to oxygen and avoiding hypoxia. To determine the effects of the SpO_2 targets, long-term (follow up) studies are needed. However, to study the effects of different SpO_2 targets, current clinical practice must be able to control the SpO_2 adequately, and follow the targets with as little deviation as possible.

There are a few drawbacks to this study. Compliance with local protocol to start resuscitation of infants > 28 weeks GA was low (57%). It was performed in a single centre and it is unclear to what extent the results are representative of other centres. Patients stayed within the NICU limits 68% (54-86) of the time, which is similar to the results of studies with dedicated clinicians adjusting the FiO_2 in the NICU[10 11 30].

Other new technological developments may help improve SpO_2 control. Providing the physician with constant feedback on deviations from the target SpO_2 could improve performance during resuscitation. With the improvement of pulse oximeters, which can provide measurements even when the infant has poor perfusion, comes a need to better understand how perfusion influences tissue oxygenation and how it changes after birth. But it will remain important to not overload the physician with information and devices to look at, as this takes the focus away from the infant. Closed loop SpO_2 control is available for use in a NICU setting ($CliO_2$, CareFusion, San Diego, USA)[31]. Similar technology might be beneficial during resuscitation, as it would keep the physician free to focus on the infant, instead of fine-tuning the equipment.

In conclusion, in our institution, the SpO_2 targets were not always followed accurately during the initial minutes after birth. At the start of resuscitation, deviations were most likely caused by an inability to control the SpO_2, i.e., no lung aeration and/or no initial SpO_2 measurement, resulting in low SpO_2 values. Whereas after the infants were stabilized, the deviations were due to weaning, pauses in respiratory support (i.e., intubation), and/or overexposure to oxygen. The ERC advise acceptable SpO_2 targets, which leaves it to the individual physician to decide how much deviation is acceptable. By changing the SpO_2 targets to a target range that depicts the acceptable deviation the targets could aid physicians in providing better respiratory support, and possibly reduce variation.

REFERENCES

1. Cunningham S, McIntosh N, Fleck B, Elton R. Transcutaneous oxygen levels in retinopathy of prematurity. *The Lancet* 1995;346(8988):1464-65.
2. Saugstad OD. Oxidative stress in the newborn--a 30-year perspective. *Biol Neonate* 2005;88(3):228-36.
3. McColm JR, Cunningham S, Wade J, Sedowofia K, Gellen B, Sharma T, et al. Hypoxic oxygen fluctuations produce less severe retinopathy than hyperoxic fluctuations in a rat model of retinopathy of prematurity. *Pediatric research* 2004;55(1):107-13.
4. Saugstad OD. Update on oxygen radical disease in neonatology. *Current Opinion in Obstetrics and Gynecology* 2001;13(2):147-53.
5. Vento M, Cheung PY, Aguar M. The first golden minutes of the extremely-low-gestational-age neonate: a gentle approach. *Neonatology* 2008;95(4):286-98.
6. Richmond S, Wyllie J. European Resuscitation Council Guidelines for Resuscitation 2010 Section 7. Resuscitation of babies at birth. *Resuscitation* 2010;81(10):1389-99.
7. Kattwinkel J, Perlman JM, Aziz K, Colby C, Fairchild K, Gallagher J, et al. Neonatal resuscitation: 2010 American Heart Association guidelines for cardiopulmonary resuscitation and emergency cardiovascular care. *Pediatrics* 2010;126(5):e1400-13.
8. Airway management and mask ventilation of the newborn infant. ARC and NZRC Guideline 2010. *Emerg Med Australas* 2011;23(4):428-35.
9. Dawson JA, Kamlin CO, Vento M, Wong C, Cole TJ, Donath SM, et al. Defining the Reference Range for Oxygen Saturation for Infants After Birth. *Pediatrics* 2010;125(6):e1340-7.
10. Bhutani VK, Taube JC, Antunes MJ, Delivoria Papadopoulos M. Adaptive control of inspired oxygen delivery to the neonate. *Pediatric pulmonology* 1992;14(2):110-17.
11. Automatic control of neonatal fractional inspired oxygen; 1991. IEEE.
12. van der Eijk AC, Dankelman J, Schutte S, Simonsz HJ, Smit BJ. An Observational Study to Quantify Manual Adjustments of the Inspired Oxygen Fraction in Extremely Low Birth Weight Infants. *Acta Paediatrica* 2011;101(3):e97-104.
13. Hagadorn JI, Furey AM, Nghiem TH, Schmid CH, Phelps DL, Pillers DAM, et al. Achieved versus intended pulse oximeter saturation in infants born less than 28 weeks' gestation: the AVIOx study. *Pediatrics* 2006;118(4):1574-82.
14. Stark A. Levels of neonatal care. *Pediatrics* 2004;114(5):1341-7.
15. Nolan J, Soar J, Zideman DA, Biarent D, Bossaert LL, Deakin C, et al. *Richtlijnen Reanimatie 2010 In Nederland en België*. Uden: Nederlandse Reanimatie Raad, 2011.
16. Escrig R, Arruza L, Izquierdo I, Villar G, Saenz P, Gimeno A, et al. Achievement of targeted saturation values in extremely low gestational age neonates resuscitated with low or high oxygen concentrations: a prospective, randomized trial. *Pediatrics* 2008;121(5):875-81.
17. Vento M, Moro M, Escrig R, Arruza L, Villar G, Izquierdo I, et al. Preterm Resuscitation With Low Oxygen Causes Less Oxidative Stress, Inflammation, and Chronic Lung Disease. *Pediatrics* 2009;124(3):e439-49.
18. Saugstad OD, Ramji S, Soll RF, Vento M. Resuscitation of newborn infants with 21% or 100% oxygen: an updated systematic review and meta-analysis. *Neonatology* 2008;94(3):176-82.
19. Mariani G, Dik PB, Ezquer A, Aguirre A, Esteban ML, Perez C, et al. Pre-ductal and Post-ductal O_2 Saturation in Healthy Term Neonates after Birth. *The Journal of pediatrics* 2007;150(4):418-21.
20. Finer N, Leone T. Oxygen saturation monitoring for the preterm infant: the evidence basis for current practice. *Pediatr Res* 2009;65(4):375-80.
21. Rook D, Schierbeek H, van der Eijk A, Longini M, Buonocore G, Vento M, et al. Resuscitation of very preterm infants with 30% vs. 65% oxygen at birth: study protocol for a randomized controlled trial. *Trials* 2012;13(1):65.
22. Saraswat A, Simionato L, Dawson JA, Thio M, Kamlin CO, Owen L, et al. Determining the best method of Nellcor pulse oximeter sensor application in neonates. *Acta Paediatr* 2012;101(5):484-7.

23. Morley CJ, Davis PG. Advances in neonatal resuscitation: supporting transition. *Archives of disease in childhood. Fetal and neonatal edition* 2008;93(5):F334-6.
24. van der Eijk AC, Horsch S, Eilers PHC, Dankelman J, Smit BJ. "New-Generation" Pulse Oximeters in Extremely Low-Birth-Weight Infants: How Do They Perform in Clinical Practice? *The Journal of Perinatal & Neonatal Nursing* 2012;26(2):172-80.
25. Leone T. Improving assisted ventilation immediately after birth. *J Pediatr* 2012;160(3):359-60.
26. Schilleman K, Witlox RS, Lopriore E, Morley CJ, Walther FJ, te Pas AB. Leak and obstruction with mask ventilation during simulated neonatal resuscitation. *Archives of disease in childhood. Fetal and neonatal edition* 2010;95(6):F398.
27. Schmölzer G, Dawson J, Kamlin C, O'Donnell C, Morley C, Davis P. Airway obstruction and gas leak during mask ventilation of preterm infants in the delivery room. *Archives of disease in childhood. Fetal and neonatal edition* 2010;96(4):f254-7.
28. Noori S, Wlodaver A, Gottipati V, McCoy M, Schultz D, Escobedo M. Transitional Changes in Cardiac and Cerebral Hemodynamics in Term Neonates at Birth. *The Journal of pediatrics* 2012;160(6):943-8.
29. Dawson JA, Vento M, Finer NN, Rich W, Saugstad OD, Morley CJ, et al. Managing Oxygen Therapy during Delivery Room Stabilization of Preterm Infants. *J Pediatr* 2011;160(1):159-61.
30. Claure N, Gerhardt T, Everett R, Musante G, Herrera C, Bancalari E. Closed-loop controlled inspired oxygen concentration for mechanically ventilated very low birth weight infants with frequent episodes of hypoxemia. *Pediatrics* 2001;107(5):1120-4.
31. Claure N, Bancalari E, D'Ugard C, Nelin L, Stein M, Ramanathan R, et al. Multicenter Crossover Study of Automated Control of Inspired Oxygen in Ventilated Preterm Infants. *Pediatrics* 2011;127(1):e76-83.

Part III

Nutritional modulation of oxidative stress

Chapter 7

Glutathione synthesis rates in early postnatal life

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ABSTRACT

Introduction

In preterm infants, antioxidant defenses are diminished. Glutathione (GSH), the main intracellular antioxidant, increases upon amino acid (AA) administration in preterm infants, without an accompanying rise of the fractional synthesis rate of GSH (FSR_{GSH}). The objective of this study was to investigate the mechanism behind this increased GSH concentration

Methods

GSH synthesis was measured either on postnatal day 1 or 2 using stable isotope techniques in very low birth weight (VLBW) infants receiving intravenous AAs. Advanced oxidized protein products (AOPPs) were determined to quantify oxidative stress.

Results

Eighteen infants (birth weight 989 ± 241 gram, gestational age of 27⅘ ± 1⅘ weeks) were studied either on postnatal day 1 or 2 (7 or 31 hours postnatally, respectively). Concentration of GSH increased with postnatal age (1.45 ± 0.48 versus 1.99 ± 0.40 mmol/L, p=0.019). FSR_{GSH} was not significantly different, but the absolute synthesis rate of GSH (ASR_{GSH}) tended to be higher in the infants studied on day 2 (8.1 ± 2.7 versus 10.6 ± 2.4 mg/(kg•d), p=0.054). AOPP concentrations were not different between groups.

Conclusions

GSH concentration in VLBW infants increases significantly after birth. A concomitant increased synthesis rate was not found, suggesting that GSH consumption decreases upon AA administration.

INTRODUCTION

The sudden increase in oxygen pressure accompanying birth results in increased formation of reactive oxygen species (ROS). If birth is marked by a period of ischemia followed by reoxygenation (e.g. asphyxia), ROS formation is further augmented through the hypoxanthine-xanthine oxidase pathway[1 2]. In addition, preterm infants are frequently exposed to ventilation with high concentrations of oxygen, further adding to this ROS formation[3]. Therefore, newborn infants, and especially preterm infants, are exposed to increased levels of ROS.

In term infants, antioxidant defenses are sufficiently present at birth to counteract this hyperoxic challenge, since antioxidant enzymes mature during late gestation[4]. Several weeks prior to birth, parallel with the rapid rise in lung surfactant, there is a 150-200% increase in superoxide dismutase and glutathione peroxidase[5 6]. Also, the transfer of several antioxidants across the placenta increases during the last days of pregnancy[4]. Thus, when an infant is born premature, most of its antioxidant defense mechanisms function suboptimally at birth. The resulting redox imbalance promotes oxidative stress, which is thought to be instrumental in the pathogenesis of the so-called 'Oxygen Radical Disease in Neonatology'. The latter comprises diseases such as bronchopulmonary dysplasia (BPD) and periventricular leukomalacia (PVL)[7].

Glutathione (GSH) is the most important intracellular antioxidant. GSH is a tripeptide consisting of the amino acids (AA) glutamate, cysteine and glycine. It is synthesized in virtually every tissue, but is mainly produced in liver and erythrocytes with erythrocytic concentrations being in the millimolar range[8]. Erythrocytes are suggested to function as antioxidant defense by being a physiological source of GSH and by taking up ROS[9 10].

While preterm infants show diminished availability of other components of the antioxidant defense systems, the GSH concentrations in cord blood of preterm infants at birth exceed those of term infants[9 11 12]. GSH concentrations, however, fall rapidly after birth in preterm infants. Recently, we demonstrated that administering AAs to preterm infants from birth onwards results in higher GSH concentrations on day 2, compared to levels found in infants receiving glucose only[13]. The purpose of the present study was to reveal the mechanism behind the increased availability of GSH with early administration of AAs. We therefore hypothesized that GSH synthesis is already upregulated very shortly after birth, resulting in the increased GSH concentration on postnatal day 2. To this aim we conducted a stable isotope technique to determine glutathione synthesis rates in the first days after birth in infants receiving AAs directly following birth. With stable isotope techniques, we are able to calculate the fractional synthesis rate (FSR_{GSH}), which is the fraction of the total intraerythrocytic GSH pool that is renewed per unit of time, and the absolute synthesis rate (ASR_{GSH}), which is the absolute amount of GSH that is produced per unit of time.

In addition, we quantified oxidative stress by determining concentrations of the advanced oxidized protein products (AOPP), first described as a marker for protein oxidation in uremic patients[14]. Hypoxic preterm infants showed higher plasma concentrations of AOPP than

normoxic preterm infants and concentrations correlated with plasma levels of hypoxanthine, which is considered a reliable marker of oxidative stress[15].

SUBJECTS AND METHODS

This study was designed as a prospective observational clinical trial in which infants were randomized to be studied either on postnatal day 1 (early group) or 2 (late group). Since this study was performed on subsequent days, different infants were used for the tracer infusion study either early or late after birth, because of the required washout time of [1-^{13}C]glycine.

The study was performed at the neonatal intensive care unit of the Erasmus MC – Sophia Children's Hospital, Rotterdam, the Netherlands. The study was investigator initiated with no funding from industry. The Erasmus MC Medical Ethical Review Board approved the protocol and informed written parental consent was obtained prior to the study.

Subjects

Preterm infants with a birth weight <1500 g, who had an indwelling arterial catheter for blood sampling and who were completely dependent on parenteral nutrition for the first 2 days of life, were eligible for this study. Exclusion criteria included receiving erythrocyte transfusions during or prior to the study, known congenital abnormalities, chromosome defects, and metabolic, endocrine, renal, or hepatic disorders.

Patients received glucose and 2.4 g of AA/(kg•d) (Primene 10%, Baxter, Clintec Benelux N.V., Brussels, Belgium) intravenously, starting within 2 hours after birth. AAs and glucose solutions were infused constantly without interruptions during the study. The AA solutions did not contain riboflavins, which are known to generate H_2O_2 when exposed to light[16]. Lipids were not administered until the end of the study period.

Birth weight, gestational age, birth weight Z-scores, antenatal corticosteroid usage, and severity of illness at entry of the study by means of Apgar and CRIB scores (clinical risk index for babies)[17] were recorded for all infants. Furthermore, blood gases, plasma AA concentrations, dependence on supplemental oxygen (expressed as the median (min-max) FiO_2 from birth until the end of the study), caloric intake and AA intake were recorded.

Tracer infusion protocol

Patients received a primed (40 µmol/kg), continuous (20 µmol/(kg•h)) infusion of [1-^{13}C]glycine during 6 hours either on day 1 (early) or on day 2 (late) . [1-^{13}C]Glycine (99% enriched, sterility and pyrogenicity tested) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and was diluted with a 0.9% saline solution by the hospital's pharmacy after which it was again tested for sterility and pyrogenicity. Tracers were infused with a Perfusor fm infusion pump (B|Braun Medical B.V., Oss, the Netherlands) along the same infusion route as the parenterally

administered nutrients. Blood was sampled from an indwelling arterial catheter after 4, 5, and 6 hours and collected in microtainers containing EDTA to quantify FSR_{GSH} in erythrocytes. Blood samples were immediately put on melting ice, after which they were centrifuged at 3500 x g for 10 min at 4°C. The plasma fraction was removed and stored separately for measurement of oxidative stress markers. The lower layer, containing primarily erythrocytes, was reconstituted to its original volume with ice-cold distilled water. The plasma fraction and cell fraction were subsequently stored at -80°C until further analysis.

Glutathione enrichments and concentration

Enrichments of GSH and its precursor glycine as well as GSH concentrations were determined to calculate fractional and absolute synthesis rates. For this purpose, a recently described technique, based on a LC-Isolink interface (Thermo Electron, Bremen, Germany) coupled to a Delta XP isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany) (LC-IRMS) was used[18]. This highly sensitive method requires only a very small sample volume, and does not require derivatization.

The concentration of GSH in human blood erythrocytes was measured by LC-IRMS using an internal standard method as described previously[18].

Calculations

The FSR_{GSH} represents the fraction of the total intraerythrocytic GSH pool that is renewed per unit of time, and is expressed as %/d.

$$FSR_{GSH}\,(\%/d) = \frac{\text{slope } E_{[1\text{-}^{13}C]GSH_{t4,5,6}}}{E_{\text{intraerythrocystic}[1\text{-}^{13}c]\text{glycine}}} \times 24h \times 100\%$$

where E stands for enrichment expressed as mole percent excess (MPE). The nominator (product) of this equation represents the hourly increase in [1-^{13}C]glycine bound GSH as calculated from the increase in enrichment between 4 and 6 hours of infusion. The denominator (precursor) represents the intraerythrocytic 1-^{13}C enrichment of free glycine at isotopic steady state. A steady-state plateau was defined as an insignificant change in time in intraerythrocytic enrichment.

Subsequently, the intravascular ASR_{GSH}, which represents the absolute amount of GSH that is produced per unit of time (mg/(kg·d)), can be calculated using the following equation:

$$ASR_{GSH}\,(mg/(kg \bullet d) = FSR_{GSH}/(100 \times \text{conc.} \times 307 \times ht \times 0.075)$$

where conc. is GSH concentration in mmol/L packed erythrocytes, 307 is the molecular weight of GSH, ht is hematocrit, and 0.075 is the estimated circulating volume in a preterm neonate, expressed as L/kg.

Amino Acid concentrations

Plasma concentrations of direct GSH precursors glutamate, glycine, and cysteine (measured as cystine), and indirect precursors glutamine, methionine and serine, were determined with a Biochrom 30 amino acid analyzer, using ninhydrin detection (Biochrom Ltd, Cambridge, England).

Plasma markers of oxidative stress

We measured AOPP in plasma by the spectrophotometric assay described by Witko-Sarsat et al[14]. The AOPP were calibrated with chloramine-T solutions that absorb at 340 nm in the presence of potassium iodide. Because the absorbance of chloramine-T at 340 nm is linear up to 100 µmol/L, AOPP concentrations were expressed as µmol/L chloramine-T equivalents.

Statistics

Statistical analyses were performed using SPSS v14.0 (SPSS Inc, Chicago, IL, USA) and Graph Prism v4 (GraphPad Software Inc, California, USA). Data are expressed as means ± SD or as medians (min – max). Primary outcome of this study was the glutathione fractional synthesis rate. Power calculation shows that, in order to detect a difference in FSR_{GSH} of 10%/d and a SD of 6% (based on our earlier study[13]) with an α of 0.05 and a power of 0.80, group size needed to be eight. We included 8 and 10 infants in each group. Differences between groups were determined using independent t-tests or Mann-Whitney tests in case of normal or skewed distribution of the population respectively. For differences in frequency of mode of delivery, Chi-Square test was used. A p value of <0.05 was considered as statistically significant.

RESULTS

The stable isotope infusion was started either on day 1 (7 ± 4.8 hours after birth, early group) or day 2 (31 ± 5.9 hours after birth, late group). Clinical characteristics are displayed in **Table 1**. The maximum inspired oxygen fraction was significantly higher in the group of infants measured on day 2. Nutritional intakes before and during the study are shown in **Table 2**.

Table 1: Clinical characteristics.

		Early group	Late group
N (male : female)		8 (7:1)	10 (8:2)
Gestational age (weeks)		28⅔ ± ⅔	27⅔ ± 2⅔
Birth weight (g)		1023 ± 180	961 ± 288
Birth weight Z-score		-0.8 ± 1.1	-1.0 ± 1.6
Mode of delivery (vaginal : cesarean section)		0:8	5:5 *
Apgar score		9 (8-10)	9 (4-9)
CRIB score[§]		2 (1-4)	4 (1-10)
Cord blood pH		7.28 ± 0.07	7.27 ± 0.17
Cord blood BE (mmol/L)		-5.1 (-6.6 – -1)	-2.5 (-22 – 1.6)
FiO_2	**minimum (%) on day 1**	21 (21-25)	21 (21-21)
	maximum (%) on day 1	30 (21-45)	55 (29-100) *
FiO_2	**minimum (%) on day 1 + 2**	21 (21-21)	21 (21-21)
	maximum (%) on day 1 + 2	31 (21-80)	58 (29-100) *

Values represent either mean ± SD or median (min-max). Mode of delivery and maximum FiO_2 were significantly different between groups. Other characteristics were not different between groups
* $p < 0.05$. [§]The CRIB score (Clinical Risk Index for Babies) indicates the degree of illness[17]. The score has a maximum of 23 points and is positively correlated with the severity of illness.

Table 2: Nutritional intakes on each study day. As anticipated, infants received increased caloric intake on day 2 (late group) compared to day 1 (early group).

	Early group	Late group
Nonprotein energy intake (kcal/(kg·d))	30 ± 4	40 ± 12 *
Amino acid intake (g/(kg·d))	2.3 ± 0.5	2.4 ± 0.1

Values are expressed as mean ± SD (Student's T-test). * $p < 0.05$.

Concentrations of plasma precursor AAs

Table 3 shows the plasma concentrations of all AAs involved in GSH synthesis with reference values obtained from healthy term breast-fed infants[19]. Cystine concentration in the late group was significantly lower than in the early group and was below the reference value[19]. Concentrations of glutamate, glutamine, methionine and serine were not different between the groups, but glutamate concentrations were below reference values in both groups.

GSH concentrations and synthesis rates

Free intraerythrocytic [1-^{13}C]glycine enrichments reached a plateau after 4 hours of infusion. Enrichments did not differ between the groups (mean 3.9 ± 0.9 and 3.4 ± 0.5 MPE in the early and late group, respectively, $p = 0.18$).

GSH kinetic data are shown in **Figure 1**. The concentration of erythrocyte GSH was significantly higher in the late group. However, FSR_{GSH} was not different between groups. Also, the ASR_{GSH} did not differ significantly between the groups (p=0.054).

Table 3: Plasma AA concentrations in µmol/L.

	Term healthy infants[§]	Preterm Early group (n=8)	Preterm Late group (n=9)
Methionine	21 – 50	46 ± 17	51 ± 19
Cystine	33 – 55	51 ± 11	31 ± 11 *
Glutamate	76– 551	66 ± 27	77 ± 46
Glutamine	147 – 623	657 ± 270	636 ± 114
Glycine	66 – 432	386 ± 166	344 ± 54
Serine	79 –227	214 ± 94	194 ± 53

Values are expressed as mean ± SD (Student's T-test). AA concentrations for one patient of the day 2 group could not be determined because of shortage of plasma. [§]Reference levels of plasma amino acid concentrations in healthy term breast-fed infants[19]. * $p < 0.002$.

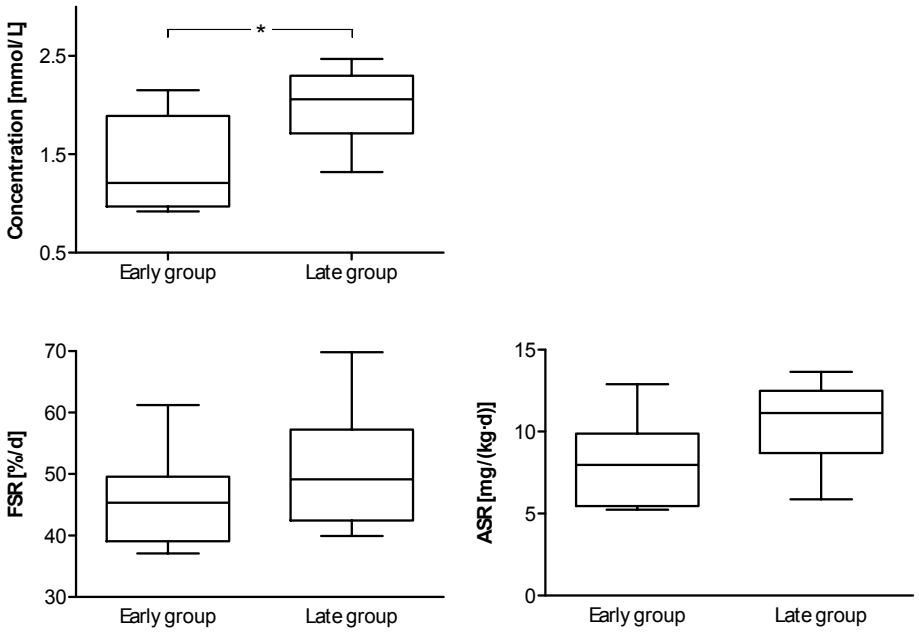

Figure 1: Glutathione concentration and synthesis rates.
Glutathione concentration, fractional synthesis rate (FSR) and absolute synthesis rates (ASR) in erythrocytes early (n=8) and late group (n=10) expressed as box-plots. Glutathione concentrations were significantly higher in the late group compared to the early group ($p<0.05$, Student's T-test). There were no significant differences in FSR and ASR between groups (Student's T-test).

Plasma levels of AOPP

Plasma AOPP levels are shown in **Figure 2**. No differences were found between groups for plasma concentration of AOPP. The mean plasma concentration of AOPP of both groups was 217 ± 108 µmol/L, which is in agreement with earlier studies on AOPP levels in preterm infants and indicates the presence of oxidative stress as compared to term infants[15 20 21].

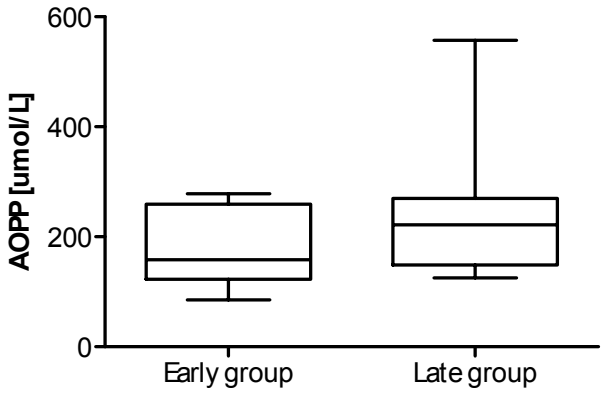

Figure 2: Plasma concentrations of advanced AOPP.
Plasma concentrations of advanced AOPP (expressed as µmol/L chloramine-T equivalents) in preterm infants early (n=8) and late (n=10) after birth. Data are expressed as box-plots. There were no statistically significant differences in AOPP concentrations between groups (Student's T-test).

DISCUSSION

In preterm infants, there is a rapid decline of GSH immediately after birth, possibly as a result of higher oxidative stress after birth. Recently, we demonstrated that early AA administration directly after birth leads to an increased GSH concentration on postnatal day 2[13]. In this study, preterm infants receiving AAs from birth onwards showed an age-related rise in GSH concentration without a concomitant rise in GSH synthesis rate, despite a higher inspired oxygen fraction in the late group.

The ASR_{GSH} tended to be higher in the infants studied on day 2 (p=0.054) with a difference of 2.5 mg/(kg·d), but this was not significantly different. Power calculation based on ASR_{GSH} showed that at least 4 infants in each group should have been included to reach a physiologic difference of 4.5 mg/(kg•d). In this study, 8 and 10 infants were included and therefore, this study has enough power. When speculating on a potential difference in ASR with an equal FSR, the observed difference in ASR would probably be almost exclusively attributed to the difference in concentration.

Since no differences were found in GSH synthesis rates, it would seem that the increased GSH concentration accompanying early AA administration is not caused by increased synthesis, suggesting that GSH consumption decreases upon AA administration.

Intracellular GSH concentration is determined by de novo synthesis, recycling of GSSG (oxidized, dimeric form of GSH) back to GSH, transport to the extracellular space, and utilization by peroxides, transferases, transhydrogenases, and transpeptidases[22]. The method for measuring GSH concentration in the present study does not discriminate between reduced and oxidized GSH. Therefore, the increased total GSH concentration in the late group cannot be explained

by different recycling rates between the groups. Moreover, recycling of oxidized GSSG back to GSH seems to be enhanced rather than decreased in preterm infants [23] [24]. Consequently, the increased GSH concentration is likely to be caused by decreased consumption of GSH after early AA administration in preterm infants.

There are several possible explanations for this decreased GSH consumption upon AA administration. First, AAs can serve as antioxidants themselves. A study in healthy elderly people, demonstrated reduced oxidative stress after the administration of essential AAs, including methionine and cysteine (both sulfur AAs)[25]. It is possible that the latter two AAs were responsible for the antioxidant effect. Previous studies indeed showed that methionine residues may protect proteins from critical oxidative damage[26], and that oral supplements with whey proteins, which contain high amounts of sulfur AAs, increase plasma GSH levels in patients with HIV[27]. The mechanism behind this increase in plasma GSH remains to be elucidated, as kinetic studies were not performed. Another possible explanation is that increased availability of AAs, as shown by increased plasma concentrations[13], up-regulates synthesis of other antioxidants. Van den Akker *et al* showed that AA administration to preterm infants results in increased albumin synthesis[28] and albumin also exerts antioxidant properties[29]. Increased levels of other antioxidants might decrease the consumption of GSH, resulting in its increased concentrations and availability.

Besides antioxidant properties, GSH also functions as a cysteine reservoir. Consequently, GSH is broken down in response to shortage of cysteine and compromised protein synthesis. This follows from the observation that GSH levels become depleted if intakes of sulfur amino acids are minimal but sufficient to maintain protein synthesis at adequate levels[30] [31]. It is therefore possible that increased availability of cysteine reduces breakdown of GSH to generate free cysteine. Besides increased GSH concentration, plasma cysteine concentrations were also increased in infants receiving AAs from birth onwards[13], suggesting decreased necessity for GSH breakdown to release cysteine. In the present study, however, plasma cysteine (measured as cystine) concentrations dropped in the first day after birth resulting in lower plasma cysteine concentrations in the late group, whereas erythrocytic glutathione concentrations had increased. Although plasma and erythrocytes represent two compartments, this decrease suggests that serving as a substrate for GSH synthesis is the metabolic fate of cysteine, most likely due to increased requirements imposed by extra uterine life. It is recently demonstrated, however, that a further increase in cysteine intake did not result in an increased GSH synthesis[32]. Alternatively, cysteine might be used for synthesis of important proteins such as albumin.

AOPP concentrations typically increase significantly in the first week in preterm infants, both in hypoxic and non-hypoxic infants[20]. In the present study, however, plasma AOPP concentrations were not yet increased in the late group, even though the maximum inspired oxygen fraction was significantly higher in this group. It might well be that antioxidant defense mechanisms, like the increased availability of GSH on day 2, avert some of the oxidative stress in preterm infants after birth.

There are some limitations in this study. First, it would have been useful to quantify oxidative stress in a broader perspective, going beyond protein oxidation only. For example, determining isoprostanes as a marker for lipid peroxidation[33]. Furthermore, the ratio between erythrocytic reduced and oxidized GSH is also suggested to be an excellent marker for oxidative stress and is already used to this aim in infants[34]. Regrettably, we could not obtain enough plasma for determining these markers, as a consequence of limited possibilities of blood withdrawal in these preterm infants. Second, the proportion of cesarean sections in the early group was significantly higher than that in the late group. It is argued that the mode of delivery might influence the degree of oxidative stress in the neonate, but literature on this topic is contradictory. Some authors report increased, while others report diminished oxidative stress[35-38].

At first glance, it can be said that the ranges of the FSR appear wide. The ranges are not caused by outliers, but are caused by variation of glutathione FSR within the population. Although the range of fractional synthesis rates is between 30-70%/day, the standard deviation is low in both groups as compared to similar studies conducted by others[8 39 40]

In this study, we demonstrate an increase of GSH concentration in the first days after birth in preterm infants receiving AAs, without a concomitant rise in GSH synthesis. Normally, glutathione is rapidly depleted after birth in preterm infants, possibly as a consequence of increased oxidative stress postnatally. Whether AA could serve as antioxidants themselves, promote synthesis of other antioxidants and thereby reducing the need for glutathione as an antioxidant or whether the increased plasma cysteine concentrations[13] decrease GSH breakdown to release free cysteine remains to be clarified.

REFERENCES

1. Richmond S, Goldsmith JP. Air or 100% oxygen in neonatal resuscitation? *Clinics in perinatology* 2006; 33(1):11-27.
2. Nemeth I, Boda D. Xanthine oxidase activity and blood glutathione redox ratio in infants and children with septic shock syndrome. *Intensive Care Med* 2001;27(1):216-21.
3. Shoji H, Koletzko B. Oxidative stress and antioxidant protection in the perinatal period. *Curr Opin Clin Nutr Metab Care* 2007;10(3):324-8.
4. Friel JK, Friesen RW, Harding SV, Roberts LJ. Evidence of oxidative stress in full-term healthy infants. *Pediatr Res* 2004;56(6):878-82.
5. Munoz-Hoyos A, Bonillo-Perales A, Avila-Villegas R, Gonzalez-Ripoll M, Uberos J, Florido-Navio J, et al. Melatonin Levels during the First Week of Life and Their Relation with the Antioxidant Response in the Perinatal Period. *Neonatology* 2007;92(3):209-16.
6. Saugstad OD. The oxygen radical disease in neonatology. *Indian J Pediatr* 1989;56(5):585-93.
7. Saugstad OD. Oxygen radical disease in neonatology. *Seminars in neonatology : SN* 1998;3:229-38.
8. Darmaun D, Smith SD, Sweeten S, Sager BK, Welch S, Mauras N. Evidence for accelerated rates of glutathione utilization and glutathione depletion in adolescents with poorly controlled type 1 diabetes. *Diabetes* 2005;54(1):190-6.
9. Ahola T, Levonen AL, Fellman V, Lapatto R. Thiol metabolism in preterm infants during the first week of life. *Scand J Clin Lab Invest* 2004;64(7):649-58.
10. Giustarini D, Milzani A, Dalle-Donne I, Rossi R. Red blood cells as a physiological source of glutathione for extracellular fluids. *Blood Cells Mol Dis* 2007;40(2):174-9.
11. Jain A, Mehta T, Auld PA, Rodrigues J, Ward RF, Schwartz MK, et al. Glutathione metabolism in newborns: evidence for glutathione deficiency in plasma, bronchoalveolar lavage fluid, and lymphocytes in prematures. *Pediatr Pulmonol* 1995;20(3):160-6.
12. Jean-Baptiste D, Rudolph N. Sequential postnatal changes in erythrocyte glutathione and sulfhydryl content: a possible adaptational response to the extrauterine environment. *Biol Neonate* 2003;84(2): 142-6.
13. Te Braake FW, Schierbeek H, de Groof K, Vermes A, Longini M, Buonocore G, et al. Glutathione synthesis rates after amino acid administration directly after birth in preterm infants. *Am J Clin Nutr* 2008; 88(2):333-9.
14. Witko-Sarsat V, Friedlander M, Capeillere-Blandin C, Nguyen-Khoa T, Nguyen AT, Zingraff J, et al. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int* 1996; 49(5):1304-13.
15. Buonocore G, Perrone S, Longini M, Terzuoli L, Bracci R. Total hydroperoxide and advanced oxidation protein products in preterm hypoxic babies. *Pediatr Res* 2000;47(2):221-4.
16. Laborie S, Lavoie JC, Pineault M, Chessex P. Contribution of multivitamins, air, and light in the generation of peroxides in adult and neonatal parenteral nutrition solutions. *Ann Pharmacother* 2000;34(4): 440-5.
17. The CRIB (clinical risk index for babies) score: a tool for assessing initial neonatal risk and comparing performance of neonatal intensive care units. The International Neonatal Network. *Lancet* 1993; 342(8865):193-8.
18. Schierbeek H, Te Braake F, Godin JP, Fay LB, van Goudoever JB. Novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2007;21(17):2805-12.
19. Wu PY, Edwards N, Storm MC. Plasma amino acid pattern in normal term breast-fed infants. *J Pediatr* 1986;109(2):347-9.
20. Buonocore G, Perrone S, Longini M, Vezzosi P, Marzocchi B, Paffetti P, et al. Oxidative stress in preterm neonates at birth and on the seventh day of life. *Pediatr Res* 2002;52(1):46-9.

21. Dani C, Giannini L, Bertini G, Pratesi S, Corsini I, Longini M, et al. Changes of nitric oxide, carbon monoxide and oxidative stress in term infants at birth. *Free Radic Res* 2007;41(12):1358-63.
22. Valencia E, Marin A, Hardy G. Glutathione--nutritional and pharmacological viewpoints: part II. *Nutrition* 2001;17(6):485-6.
23. Clahsen PC, Moison RM, Holtzer CA, Berger HM. Recycling of glutathione during oxidative stress in erythrocytes of the newborn. *Pediatr Res* 1992;32(4):399-402.
24. Frosali S, Di Simplicio P, Perrone S, Di Giuseppe D, Longini M, Tanganelli D, et al. Glutathione recycling and antioxidant enzyme activities in erythrocytes of term and preterm newborns at birth. *Biol Neonate* 2004;85(3):188-94.
25. Manzella D, Grella R, Esposito K, Cacciapuoti F, Arciello A, Giugliano D, et al. Oral amino acid administration decreases oxidative stress and improves brachial reactivity in elderly individuals. *Am J Hypertens* 2005;18(6):858-63.
26. Levine RL, Berlett BS, Moskovitz J, Mosoni L, Stadtman ER. Methionine residues may protect proteins from critical oxidative damage. *Mech Ageing Dev* 1999;107(3):323-32.
27. Micke P, Beeh KM, Schlaak JF, Buhl R. Oral supplementation with whey proteins increases plasma glutathione levels of HIV-infected patients. *Eur J Clin Invest* 2001;31(2):171-8.
28. van den Akker CH, te Braake FW, Schierbeek H, Rietveld T, Wattimena DJ, Bunt JE, et al. Albumin synthesis in premature neonates is stimulated by parenterally administered amino acids during the first days of life. *Am J Clin Nutr* 2007;86(4):1003-8.
29. Halliwell B. Albumin--an important extracellular antioxidant? *Biochem Pharmacol* 1988;37(4):569-71.
30. Stipanuk MH, Coloso RM, Garcia RA, Banks MF. Cysteine concentration regulates cysteine metabolism to glutathione, sulfate and taurine in rat hepatocytes. *The Journal of nutrition* 1992;122(3):420-7.
31. Stipanuk MH, Dominy JE, Jr., Lee JI, Coloso RM. Mammalian cysteine metabolism: new insights into regulation of cysteine metabolism. *The Journal of nutrition* 2006;136(6 Suppl):1652S-59S.
32. Te Braake FW, Schierbeek H, Vermes A, Huijmans JG, van Goudoever JB. High-dose cysteine administration does not increase synthesis of the antioxidant glutathione preterm infants. *Pediatrics* 2009; 124(5):e978-84.
33. Roberts LJ, Morrow JD. Measurement of F(2)-isoprostanes as an index of oxidative stress in vivo. *Free radical biology & medicine* 2000;28(4):505-13.
34. Vento M, Asensi M, Sastre J, Lloret A, Garcia-Sala F, Minana JB, et al. Hyperoxemia caused by resuscitation with pure oxygen may alter intracellular redox status by increasing oxidized glutathione in asphyxiated newly born infants. *Seminars in perinatology* 2002;26(6):406-10.
35. Rogers MS, Mongelli JM, Tsang KH, Wang CC, Law KP. Lipid peroxidation in cord blood at birth: the effect of labour. *Br J Obstet Gynaecol* 1998;105(7):739-44.
36. Paamoni-Keren O, Silberstein T, Burg A, Raz I, Mazor M, Saphier O. Oxidative stress as determined by glutathione (GSH) concentrations in venous cord blood in elective cesarean delivery versus uncomplicated vaginal delivery. *Arch Gynecol Obstet* 2007;276(1):43-6.
37. Raijmakers MT, Roes EM, Steegers EA, van der Wildt B, Peters WH. Umbilical glutathione levels are higher after vaginal birth than after cesarean section. *J Perinat Med* 2003;31(6):520-2.
38. Inanc F, Kilinc M, Kiran G, Guven A, Kurutas EB, Cikim IG, et al. Relationship between oxidative stress in cord blood and route of delivery. *Fetal Diagn Ther* 2005;20(5):450-3.
39. Badaloo A, Reid M, Forrester T, Heird WC, Jahoor F. Cysteine supplementation improves the erythrocyte glutathione synthesis rate in children with severe edematous malnutrition. *Am J Clin Nutr* 2002; 76(3):646-52.
40. Lyons J, Rauh-Pfeiffer A, Ming-Yu Y, Lu XM, Zurakowski D, Curley M, et al. Cysteine metabolism and whole blood glutathione synthesis in septic pediatric patients. *Crit Care Med* 2001;29(4):870-7.

Chapter 8

Increased amino acid and early lipid administration do not up-regulate glutathione synthesis, nor increase oxidative stress in very low birth weight infants

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Submitted

ABSTRACT

Introduction

Preterm infants are subjected to increased oxidative stress, which is associated with bronchopulmonary dysplasia, retinopathy of prematurity and periventricular leukomalacia. Previously, we demonstrated that early amino acid (AA) administration in preterm infants increased glutathione (GSH) concentration, the main non-enzymatic intracellular antioxidant. The objective of this study was to investigate whether additional AAs and energy would further increase GSH synthesis and to investigate whether early lipid administration would increase oxidative stress.

Methods

Very low birth weight (VLBW) infants were randomized to receiving 2.4 or 3.6 g/(kg•d) AAs with or without 2 g/(kg•d) lipids from birth onwards. On day 2, infants received a primed, continuous infusion of [U-^{13}C]glycine to determine intra-erythrocyte GSH synthesis rates. Oxidative stress markers were measured in urine. Data are presented as median (interquartile range).

Results

Thirty-one VLBW infants with gestational age 27 (25⅔-28⅔) weeks and birth weight 820 (670-1000) g were included. Total GSH concentration, GSH synthesis and oxidative stress markers did not change upon increasing the AA intake to 3.6 g/(kg•d) or increasing the energy intake by 2 g/(kg•d) lipid administration from birth onwards. Total median values were: GSH concentration 1.8 (1.4-2.0) mmol/L; fractional synthesis rate 41 (37-49) %/d; and absolute synthesis rate 6.5 (5.3-8.5) mg/(kg•d).

Conclusions

Increasing AA intake to 3.6 g/(kg•d) with a concomitant increase in energy intake from birth onwards in VLBW infants did not increase GSH synthesis. Lipid infusion directly following birth did not increase oxidative stress.

INTRODUCTION

Preterm infants are highly susceptible to oxidative stress. Due to an immature antioxidant defense system[1 2], preterm infants are unable to counteract the increased production of reactive oxygen species associated with, amongst others, supplemental oxygen administration and infections[3 4]. This results in increased oxidative stress which is thought to play a role in the pathogenesis of bronchopulmonary dysplasia, necrotizing enterocolitis, retinopathy of prematurity and periventricular leukomalacia[5].

Early nutritional intake is known to have a positive effect in preterm infants, e.g. improved nitrogen balance, synthesis of specific proteins and growth, and is associated with improved neurodevelopmental outcome[6 7]. Previously, we demonstrated that immediate administration of amino acids (AA) following birth increased the concentration of glutathione (GSH), the main intracellular non-enzymatic antioxidant, without a concomitant increase of GSH synthesis[8 9]. This increased GSH availability was not associated with a decrease of oxidative stress markers, suggesting that antioxidant defenses are still suboptimal.

Non-protein energy deficits are common in preterm infants during the first week of life[10]. Optimizing early nutrition by increasing the AA intake and early administration of lipids directly following birth might further improve outcome of preterm infants. However, the immediate administration of lipids to preterm infants has been withheld frequently by concerns regarding lipid intolerance and a possible association with bronchopulmonary dysplasia[11 12]. Consequently, some physicians are still reluctant to start administration of lipids to preterm infants during the first days of life[13], although recent meta-analyses do not suggest adverse effects[14 15]. However, lipids are prone to peroxidation, which has been associated with increased oxidative stress[16 17]. In this study, we hypothesized that a higher AA intake and additional energy, provided via intravenous lipids from birth onwards, results in increased GSH synthesis, without increasing oxidative stress.

SUBJECTS AND METHODS

This randomized, clinical trial was executed at the Neonatal Intensive Care Unit of the Erasmus MC - Sophia Children's Hospital (Rotterdam, the Netherlands) between 2009 and 2011. The study protocol was approved by the institutional medical ethical review board. Parental informed consent was obtained prenatally or within a few hours after birth.

Subjects

Inborn very low birth weight (VLBW; birth weight <1500g) infants with a central venous and arterial catheter for clinical purposes were eligible for the study. Exclusion criteria were congenital anomalies, including chromosome defects, and infants with metabolic diseases, or

endocrine, renal or hepatic disorders. The present study was part of a larger study testing the hypothesis that anabolism, as measured by nitrogen balance, would significantly improve by either additional energy alone (+18 kcal/(kg•d)) or by additional energy (+18 kcal/(kg•d)) and additional AAs (+1.2 g/(kg•d)).

Randomization and masking

Within 6 hours after birth, the attending physician included the infant by opening a sealed opaque randomization envelope, stratified for birth weight (<1000g and 1000-1499 g) and sex. Envelopes were prepared by a research-pharmacist, who was not involved in clinical care, based on a computer-generated block randomization list with variable block sizes, provided by a statistician. For logistic reasons, randomization to the study group was open after inclusion. The laboratory analysts were blinded for study group randomization.

Nutritional intervention

Infants were randomized to different nutritional regiments (**Figure 1**). All infants received glucose and AAs [Primene 10%, Baxter, Deerfield, IL, USA; 2.4 g/(kg·d)] from birth onwards. In infants in the control group, lipids [Intralipid 20%, Fresenius Kabi, Bad Homburg, Germany; starting dose of 2 g/(kg·d)] were started at the end of the second postnatal day. Infants in the intervention groups received lipids from birth onwards and were randomized to Intralipid 20% or SMOFlipid 20% [Fresenius Kabi, Bad Homburg,Germany; starting dose of 2 g/(kg·d), following days 3 g/(kg·d)]. The type of lipid emulsion had no effect on our primary outcome (data not shown), so groups were pooled according to their macronutrient intake. Whenever

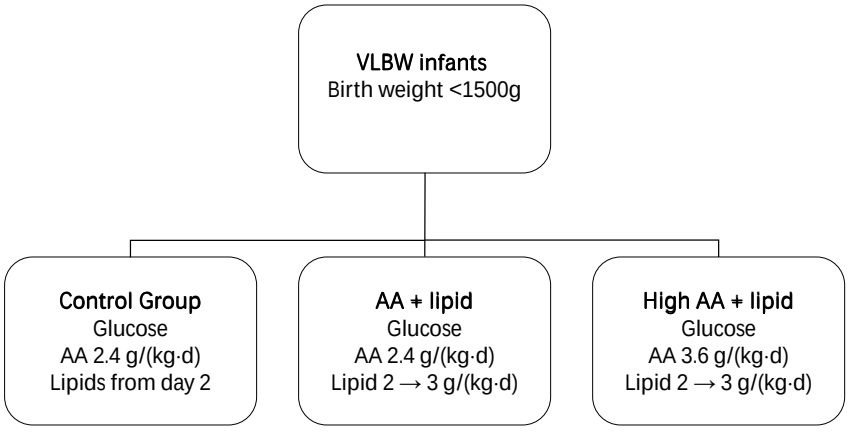

Figure 1: Schematic representation of the nutritional regiments of study groups.
VLBW = very low birth weight, AA = amino acids (Primene 10%, Baxter, Deerfield, IL, USA), lipids (Intralipid 20% or SMOFlipid 20%, Fresenius Kabi, Germany).

possible, minimal enteral feeding was initiated with either mother's milk or preterm formula on the day of birth and advanced to full enteral nutrition in the following days according to standard protocol.

Glutathione kinetics

Tracer infusion protocol and blood sampling

[U-^{13}C]glycine (99% enriched, Cambridge Isotope Laboratories, Andover, MA, USA; GMP, sterility and pyrogenicity tested) was dissolved in 0.9% saline by the hospital pharmacist according to GMP guidelines. On the second postnatal day, a primed (20 µmol/kg) continuous (20 µmol/(kg·h)) infusion of [U-^{13}C]glycine was administered intravenously during 8 hours using a Perfusor fm infusion pump (B Braun Medical B.V., Oss, the Netherlands). Blood was sampled from an indwelling arterial catheter after 6, 7 and 8 hours and collected in EDTA containing microtainers. The blood samples were immediately put on melting ice and centrifuged at 3500 x g for 10 min. The plasma fraction was removed, and the lower layer (containing primarily erythrocytes) was reconstituted to its original volume with ice-cold distilled water and stored at -80°C until further analysis. To calculate the fractional synthesis rates (FSR) and absolute synthesis rates (ASR), concentrations and enrichments of GSH and its precursor glycine were determined in the erythrocytes by means of mass spectrometry techniques.

Mass spectrometric analysis of glutathione enrichment and concentration

Analysis of GSH was performed on a LC-Isolink interface coupled to a Delta XP isotope ratio mass spectrometer (LC/IRMS) (Thermo Fisher, Bremen, Germany) using a previously described method[18]. This highly sensitive method requires only a very small sample, and does not require derivatization of the sample.

Mass spectrometric analysis of glycine enrichment

The erythrocyte enrichment of [U-^{13}C]glycine was measured by gas chromatography-mass spectrometry (GCMS) as their ethyl chloroformate (ECF) ester derivatives, using a MSD 5975C Agilent GCMS (Agilent Technologies, Amstelveen, the Netherlands). Briefly, 25 µL aliquots of the remaining supernatant used for the GSH analysis were taken, acidified by adding 50 µL of 0.1 M HCl and diluted with 125 µL of distilled water. ECF derivatization of the samples was performed according to a modified procedure of Hušek[19]. A VF-17ms column (30 m x 0.25 mm id, 0.12 µm film thickness; Varian, Middelburg, the Netherlands) was used for the separation. The samples were measured using a selected ion monitoring (SIM) method in electron impact mode. The mass fragments with a mass to charge (m/z) of 102.1 for unenriched (M) and a m/z 103.1 for the enriched (M + 1) glycine respectively, were selected for this purpose. Due to fragmentation there is a loss of the primary ^{13}C of glycine. Therefore we used the m/z 103.1 fragment as a measure for the enrichment of glycine. Each sample was analyzed in triplicate by GC/MS.

Enrichments were calculated from the mean of the triplicate analyses using a calibration graph and expressed as mole percent excess (MPE).

Calculations

The FSR_{GSH} represents the fraction of the total intraerythrocytic GSH pool that is renewed per unit of time and is expressed as %/d.

$$FSR_{GSH}\,(\%/d) = \frac{\text{slope } E_{[U\text{-}^{13}C]GSH_{t6,7,8}}}{E_{intraerythrocystic[U\text{-}^{13}c]glycine}} \times 24h \times 100\%$$

where E stands for the enrichment expressed as MPE. The nominator (product) of this equation represents the hourly increase in [U-^{13}C]glycine bound GSH, as calculated from the increase in enrichment between 6 and 8 hours of infusion. The denominator (precursor) represents the intraerythrocytic [U-^{13}C] enrichment of free glycine at isotopic steady state. A steady-state plateau was defined as an insignificant change in intraerythrocytic enrichment over time. Subsequently, the intravascular ASR_{GSH}, which represents the absolute amount of GSH that is produced per unit of time (mg/(kg×d)), can be calculated using the following equation:

$$ASR_{GSH}\,(mg/(kg \bullet d)) = FSR_{GSH}/(100 \times conc. \times 307 \times ht \times 0.075),$$

where conc. is GSH concentration in mmol/L packed erythrocytes, 307 is the molecular weight of GSH, ht is hematocrit and 0.075 is the estimated circulating volume in a preterm neonate, expressed as L/kg.

Urinary oxidative stress markers

On postnatal day 2, 4 and 6, urine samples were collected. After centrifugation at 2800 x g for 5 minutes, the samples were stored at -20°C until analysis. Urine samples were shipped on dry ice to the University & Polytechnic Hospital La Fe (Valencia, Spain), where the urinary oxidative stress markers were determined.

Urinary 8-hydroxy-2'-deoxyguanosine/2-deoxyguanosine ratio (8OhdG/2dG; marker of oxidative damage to DNA), Ortho-tyrosine/phenylalanine ratio (O-Tyr/Phe; marker of oxidative damage to protein), 3-Nitro-tyrosine (3-N-Tyr; marker of nitrosative damage to protein) and 3-Chloro-tyrosine (3-Cl-Tyr; marker of inflammation caused by free radicals) were determined by ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), modified for 3-N-Tyr and 3-Cl-Tyr based on the method previously described by Orhan et al.[20 21]. UPLC-MS/MS was carried out at the Central Service for the Support to Experimental Research (SCSIE) of the University of Valencia, using an Acquity UPLC TQDetector (Waters, Manchester, UK). Analytical separation was performed using a core shell C18 Acquity UPLC® BEH column (2.1x 50mm, 1.7 µm, Waters). Transitions (m/z) and retention times for each considered

analyte were the following, Phe (166.1>120.1; 1.7 min), O-Tyr (182.05>136.1; 1.38 min), 3-N-Tyr (227.1>181.2; 1.96 min), 3-Cl-Tyr (216.1>170.1; 1.60 min), 8OhdG (284.1>168.1; 0.91 min) and 2dG (268.1>152.1; 0.92 min).

For urinary isoprostanes, isofurans, neuroprostanes and neurofurans determinations, samples were subjected to a solid phase extraction procedure for clean-up providing a pre-concentration factor of 10. If the sample volume was too low for the direct application of the procedure, samples were diluted in H_2O prior to the extraction procedure. Chromatographic separations were carried out using a Kinetex C18 column (2.1x100, 1.7 µM, Phenomenex, USA) running a linear CH_3OH:H_2O gradient from 30% to 90% CH_3OH in 4 min at a flow of 0.4 ml min-1 and using an injection volume of 5 µL. Chromatograms for total isoprostanes, isofurans, neuroprostanes and neurofurans were recorded as described previously[22]. Results were expressed as 'total response' because, due to the lack of commercially available standards, only relative response values were available.

Statistical analysis

Statistical analysis was performed using SPSS PASW 17 (SPSS Inc, Chicago, IL, USA). Unless otherwise stated, data are expressed as medians (interquartile range). The primary outcome of this study was the FSR_{GSH}. Power calculation showed that, in order to detect a difference in FSR_{GSH} of 10%/d and a SD of 6% (based on our earlier study[8]) with an α of 0.05 and a power of 0.80, group size needed to be 7 in each group, so 21 for the whole study. For continuous data, differences between groups were determined using Kruskal-Wallis test and for categorical data, differences were determined using the Chi-Square test. A p value of <0.05 was considered as statistically significant.

RESULTS

Thirty-one VLBW infants were analyzed in this study (**Figure 2**). Due to the study design, parental informed consent had to be obtained prior to or within 6 hours of birth, which was not possible in 55 infants. After randomization to the three study groups, not all infants were eligible for further analysis. The main reasons were reduction of nutrition according to clinical protocol (i.e. plasma urea concentration ≥ 10 mmol/L or triglyceride concentration ≥ 3 mmol/L) before the start of the isotope study and clinical contra-indications for the isotope study (e.g., fluid restriction or restriction of blood withdrawals).

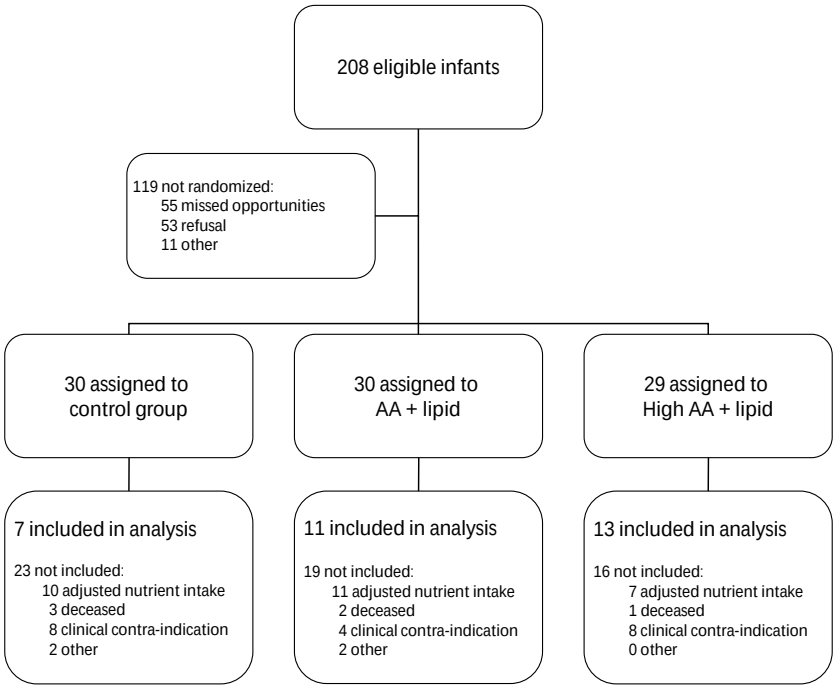

Figure 2: Trial profile.

Table 1: Clinical characteristics.

	Control	AA + lipid	High AA + lipid
N (male:female)	7 (6:1)	11 (6:5)	13 (8:5)
Gestational age (weeks)	29⅘ (27⅗ - 29⅘)	26⅘ (25 - 27⅘)	26⅘ (25⅗ - 28)
Birth weight (g)	980 (670 - 1135)	800 (630 - 1000)	760 (663 - 970)
Birth weight Z-score	-2.0 (-2.8 - -0.1)	-1.0 (-2.5 - -0.2)	-1.7 (-2.5 - -0.8)
Cord blood pH	7.21 (7.12 - 7.24)	7.30 (7.21 - 7.36)	7.30 (7.21 - 7.33)
Apgar score at 5 min	8 (4 - 9)	8 (6 - 9)	8 (6 - 9)
CRIB score[§]	4 (1 - 8)	4 (2 - 8)	4 (1 - 9)
Mortality (n)	0	2	2

Data are presented as median (interquartile range). [§]The CRIB score (Clinical Risk Index for Babies) indicates the degree of illness[23] . The score has a maximum of 23 points and is positively correlated with the severity of illness.

Demographic and study characteristics

Patient characteristics are shown in **Table 1**. Gestational age was not correlated with GSH kinetics (data not shown). Nutritional intake and oxygen administration on the study day (postnatal day 2) are displayed in **Table 2**. According to the study design, nutritional intakes were different between groups.

Plasma concentrations of AAs involved in GSH synthesis were not different between groups (**Table 3**). However, median cystine levels were below the reference values[24] in all groups, especially in the group receiving 2.4 g/(kg·d) AAs and 2 g/(kg·d) of lipids. Also, median methionine level was below reference values in that group.

Table 2: Parenteral intake and oxygen administration on postnatal day 2.

		Control	AA + lipid	High AA + lipid
Amino acid intake in g/(kg×d)		2.4 (2.3 - 2.4)	2.3 (2.3 - 2.4)	3.6 (3.5 - 3.7) #*
Non-protein intake in kcal/(kg×d)		35 (25 - 43)	54 (50 - 60) #	53 (47 - 57) #
Total energy intake in kcal/(kg×d)		44 (35 - 52)	63 (60 - 70) #	68 (62 - 71) #
FiO_2 (%)	**Minimum**	21 (21 -21)	21 (21 -24)	21 (21 -28)
	Maximum	33 (21 - 40)	34 (30 - 50)	35 (21 - 48)
	Median	21 (21 - 21)	24 (21 - 29)	23 (21 - 32)

Data are presented as median (interquartile range). # $p < 0.01$ compared with Control, * $p < 0.01$ compared with AA + lipid. FiO_2 = fraction of inspired oxygen.

Table 3: Plasma concentrations of amino acids involved in GSH synthesis (μmol/L).

	Term healthy infants[§]	Control	AA + lipid	High AA + lipid
Methionine	21 - 52	21 (17 - 42)	15 (11 - 25)	35 (22 - 42)
Cystine	33 - 55	32 (26 - 33)	20 (15 - 37)	32 (26 - 42)
Glutamate	76 - 551	24 (19 - 35)	28 (17 - 42)	73 (28 - 96)
Glutamine	147 - 623	511 (356 - 888)	408 (322 - 481)	442 (421 - 751)
Glycine	66 - 432	228 (206 - 341)	249 (189 - 297)	278 (245 - 415)
Serine	79 - 227	143 (99 - 234)	138 (130 - 197)	225 (183 - 268)

Values are expressed as median (interquartile range).[§]Reference concentrations of plasma amino acid concentrations in healthy term breast-fed infants[24].

GSH kinetics and oxidative stress markers

GSH kinetic data are presented in **Figure 3**. The concentration of total erythrocyte GSH, the FSR_{GSH} and the ASR_{GSH} were not different between groups. Cystine concentrations were not correlated with GSH kinetics (data not shown). **Figure 4** depicts the urinary oxidative stress markers. Total isofurans and neurofurans were significantly increased on postnatal day 2 in the groups receiving lipids from birth onwards (p=0.002 and p=0.01 respectively). On postnatal day 4 and 6, total isofurans and neurofurans were similar between groups. The other urinary markers of oxidative damage were not different between the groups.

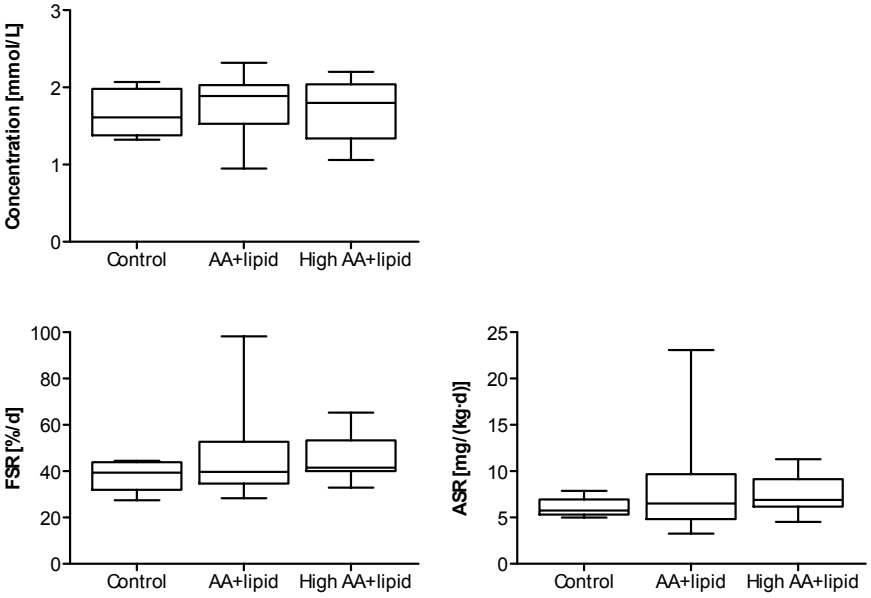

Figure 3: GSH synthesis rates and concentration on postnatal day 2.
Data are shown as box-plots. Glutathione concentration, fractional synthesis rate (FSR) and absolute synthesis rate (ASR) were similar in the groups.

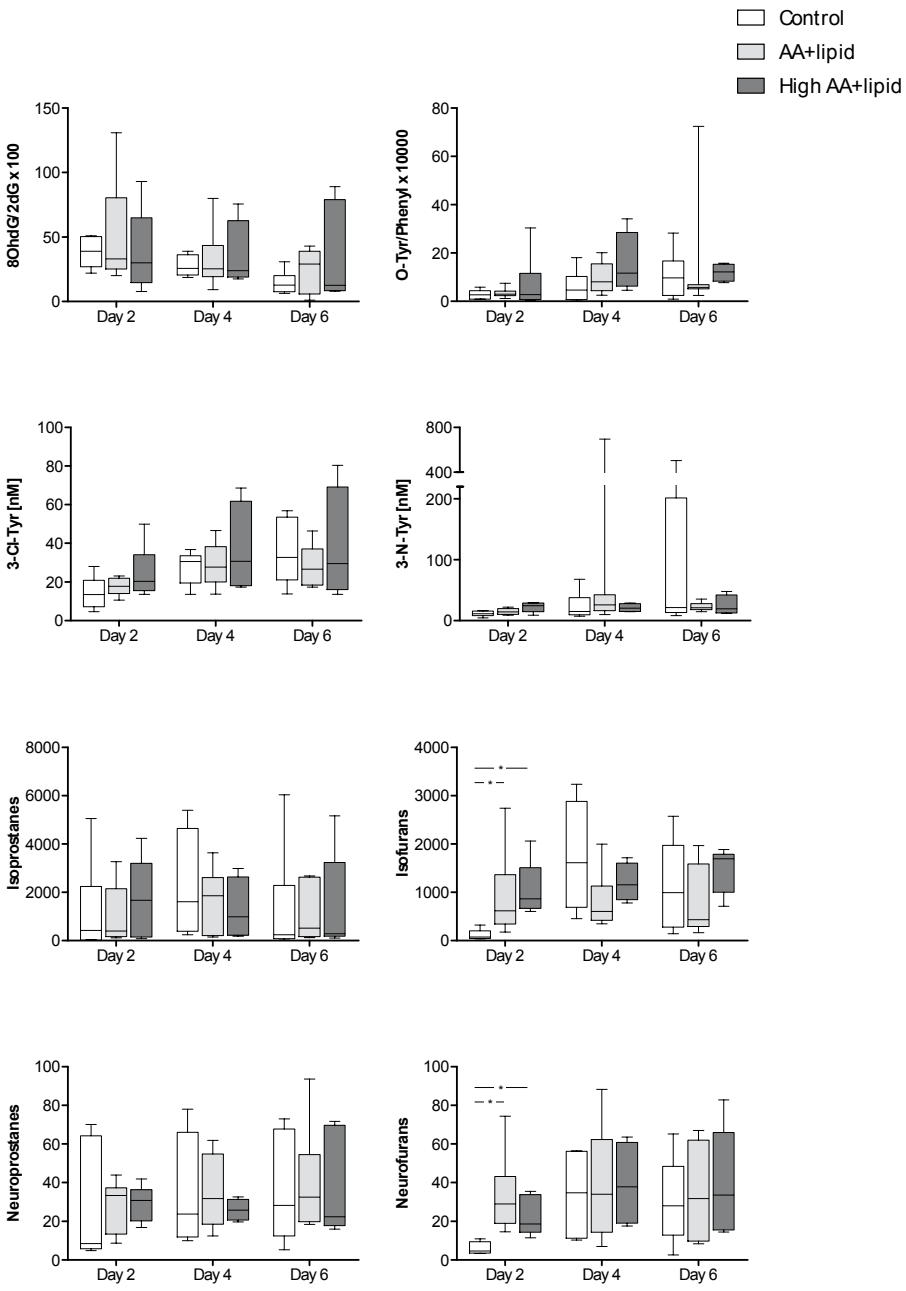

Figure 4: Urinary oxidative stress markers on postnatal day 2, 4 and 6.
Data are shown as box-plots. 8OhdG/2dG = 8-hydroxy-2'-deoxyguanosine/2-deoxyguanosine ratio, O-Tyr/Phe = Ortho-tyrosine/phenylalanine ratio, 3-N-Tyr = 3-Nitro-tyrosine, 3-Cl-Tyr = 3-Chloro-tyrosine. Total isoprostanes, isofurans, neuroprostanes and neurofurans are expressed as 'total response'. * $p < 0.05$

DISCUSSION

Increased AA and early lipid administration did not result in increased glutathione synthesis in very low birth weight infants. Although total isofurans and neurofurans were increased on postnatal day 2 upon early lipid administration, these differences disappeared in the consecutive days when all groups received lipids. Also, other oxidative stress markers were not different between groups. Therefore, it seems that lipid administration from birth onwards does not increase oxidative stress compared to initiation of lipids later.

Previously, we demonstrated that early AA administration of 2.4 g/(kg•d) directly from birth onwards resulted in increased GSH availability, without concomitant increase in GSH synthesis or decreased oxidative stress markers[8 9]. To further enhance GSH availability, we hypothesized that additional AA would increase GSH synthesis and thus decrease oxidative stress in preterm infants. However, increasing AA administration to 3.6 g/(kg•d) did not further increase GSH concentration or synthesis rate compared to starting at 2.4 g/(kg•d) in VLBW infants. Protein synthesis is an energy demanding process and a positive energy balance is thus important for protein synthesis. Since lipids become the main source of energy within hours after administration[7], early lipid administration may result in protein sparing. However, lipid administration from birth onwards did not result in increased GSH synthesis or concentration. On the other hand, early lipid administration did not result in increased oxidative stress suggesting that lipids can be administrated without increased oxidative stress.

The lack of increased GSH synthesis upon increased AA or energy intake might suggest that there is no need for upregulation of GSH synthesis in preterm infants. A higher recycling of GSSG (dimeric oxidized form of GSH) into GSH in erythrocytes was observed in preterm infants compared with adults[25]. The more efficient recycling could decrease the need for de novo GSH synthesis, since there is relatively more effective GSH. For measurement of the GSH kinetics, all of the GSH was transformed to GSSG and we did not differentiate between GSH and its dimeric form in this study. The GSH/GSSG ratio would have provided additional information and could give us an indication of the oxidative stress in these infants[26]. Unfortunately, we were not able to measure GSH and GSSG separately.

Another explanation for the lack of differences between the groups could be a maximum synthetic capacity for GSH synthesis in preterm infants, either via an immature enzymatic apparatus or lack of substrate. Glutamate-cysteine ligase - the rate-limiting enzyme in GSH synthesis - seems to be present and active in preterm infants[27]. However, literature about cystathionase is conflicting[28-31]. Cystathionase converts homocysteine to L-cysteine, which is a precursor for GSH. It has been suggested that cystathionase is not fully matured in preterm infants, making cysteine a conditionally essential AA in preterm infants. However, previously it has been shown that increased cysteine administration does not result in increased GSH synthesis[32]. Also in the present study, cysteine concentrations were not correlated with GSH synthesis. Furthermore, cysteine synthesis from methionine and serine is certainly possible in

preterm infants[33-35]. Taken together, lack of cysteine, either by cystathionase deficiency or, even less likely, insufficient substrate, does not seem to be the main determinant of GSH synthesis in preterm infants. Moreover, median FSR in this study was 41%/d but with a range of 27 - 98%/d, suggesting that GSH synthesis is not hampered in preterm infants by a maximum synthetic capacity. Interestingly, median plasma cysteine and methionine concentrations were clearly below reference range in the group receiving 2.4 g/(kg·d) AAs and 2 g/(kg·d) of lipids, whereas mean cysteine values in the other groups were just below reference range[24]. Although no correlation was found between cysteine levels and GSH kinetics, again this finding shows that the optimal AA mixture for preterm infants has not been found.

A possible limitation of this study might be a selection bias of infants. Due to clinical contraindications for the isotope study (e.g., fluid restriction or restriction of blood withdrawals), not all randomized infants could be studied. It could be speculated that the excluded infants are the most sick infants, creating a selection bias. However, this decision was made by the attending physician who was not involved in this study and this selection occurred equally in all study groups. Therefore, we assume that it is not of influence on our results.

In conclusion, administration of high amounts of AA and/or additional energy (via intravenous lipids) from birth onwards does not increase GSH concentration or synthesis. In addition, administration of lipids from birth onwards does not result in increased oxidative stress as reflected by GSH concentration and urinary oxidative stress markers. Although early initiation of lipids did not results in differences in short-term clinical outcome (Vlaardingerbroek et al., *submitted*), long-term follow-up will determine whether the transient increase of isofurans and neurofurans upon early initiation of lipids is indeed clinically irrelevant.

REFERENCES

1. Georgeson GD, Szony BJ, Streitman K, Varga IS, Kovacs A, Kovacs L, et al. Antioxidant enzyme activities are decreased in preterm infants and in neonates born via caesarean section. *Eur J Obstet Gynecol Reprod Biol* 2002;103(2):136-9.
2. Buhimschi IA, Buhimschi CS, Pupkin M, Weiner CP. Beneficial impact of term labor: nonenzymatic antioxidant reserve in the human fetus. *Am J Obstet Gynecol* 2003;189(1):181-8.
3. Comporti M, Signorini C, Leoncini S, Buonocore G, Rossi V, Ciccoli L. Plasma F2-isoprostanes are elevated in newborns and inversely correlated to gestational age. *Free radical biology & medicine* 2004;37(5):724-32.
4. Buonocore G, Perrone S, Longini M, Vezzosi P, Marzocchi B, Paffetti P, et al. Oxidative stress in preterm neonates at birth and on the seventh day of life. *Pediatr Res* 2002;52(1):46-9.
5. Saugstad OD. The oxygen radical disease in neonatology. *Indian J Pediatr* 1989;56(5):585-93.
6. van den Akker CH, Vlaardingerbroek H, van Goudoever JB. Nutritional support for extremely low-birth weight infants: abandoning catabolism in the neonatal intensive care unit. *Curr Opin Clin Nutr Metab Care* 2010;13(3):327-35.
7. Vlaardingerbroek H, van Goudoever JB, van den Akker CH. Initial nutritional management of the preterm infant. *Early Hum Dev* 2009;85(11):691-5.
8. Te Braake FW, Schierbeek H, de Groof K, Vermes A, Longini M, Buonocore G, et al. Glutathione synthesis rates after amino acid administration directly after birth in preterm infants. *Am J Clin Nutr* 2008; 88(2):333-9.
9. Rook D, Te Braake FW, Schierbeek H, Longini M, Buonocore G, van Goudoever JB. Glutathione Synthesis Rates in Early Postnatal Life. *Pediatr Res* 2010;67(4):407-11.
10. Hulst JM, Goudoever JB, Zimmermann LJI, Hop WCJ, Albers MJIJ, Tibboel D, et al. The effect of cumulative energy and protein deficiency on anthropometric parameters in a pediatric ICU population. *Clinical Nutrition* 2004;23(6):1381-89.
11. Alwaidh MH, Bowden L, Shaw B, Ryan SW. Randomised trial of effect of delayed intravenous lipid administration on chronic lung disease in preterm neonates. *J Pediatr Gastroenterol Nutr* 1996;22(3): 303-6.
12. Sosenko IR, Rodriguez-Pierce M, Bancalari E. Effect of early initiation of intravenous lipid administration on the incidence and severity of chronic lung disease in premature infants. *J Pediatr* 1993;123(6): 975-82.
13. Lapillonne A, Fellous L, Kermorvant-Duchemin E. Use of parenteral lipid emulsions in French neonatal ICUs. *Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition* 2011;26(6):672-80.
14. Vlaardingerbroek H, Veldhorst MA, Spronk S, van den Akker CH, van Goudoever JB. Parenteral lipid administration to very-low-birth-weight infants--early introduction of lipids and use of new lipid emulsions: a systematic review and meta-analysis. *Am J Clin Nutr* 2012.
15. Simmer K, Rao SC. Early introduction of lipids to parenterally-fed preterm infants. *Cochrane Database Syst Rev* 2005(2):CD005256.
16. Helbock HJ, Motchnik PA, Ames BN. Toxic hydroperoxides in intravenous lipid emulsions used in preterm infants. *Pediatrics* 1993;91(1):83-7.
17. Laborie S, Lavoie JC, Chessex P. Increased urinary peroxides in newborn infants receiving parenteral nutrition exposed to light. *J Pediatr* 2000;136(5):628-32.
18. Schierbeek H, Te Braake F, Godin JP, Fay LB, van Goudoever JB. Novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2007;21(17):2805-12.
19. Hušek P. Rapid derivatization and gas chromatographic determination of amino acids. *Journal of Chromatography A* 1991;552(0):289-99.

20. Orhan H, Vermeulen NP, Tump C, Zappey H, Meerman JH. Simultaneous determination of tyrosine, phenylalanine and deoxyguanosine oxidation products by liquid chromatography-tandem mass spectrometry as non-invasive biomarkers for oxidative damage. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004;799(2):245-54.
21. Solberg R, Andresen JH, Escrig R, Vento M, Saugstad OD. Resuscitation of hypoxic newborn piglets with oxygen induces a dose-dependent increase in markers of oxidation. *Pediatr Res* 2007;62(5):559-63.
22. Song WL, Lawson JA, Reilly D, Rokach J, Chang CT, Giasson B, et al. Neurofurans, novel indices of oxidant stress derived from docosahexaenoic acid. *The Journal of biological chemistry* 2008;283(1): 6-16.
23. The CRIB (clinical risk index for babies) score: a tool for assessing initial neonatal risk and comparing performance of neonatal intensive care units. The International Neonatal Network. *Lancet* 1993; 342(8865):193-8.
24. Scott PH, Sandham S, Balmer SE, Wharton BA. Diet-related reference values for plasma amino acids in newborns measured by reversed-phase HPLC. *Clinical chemistry* 1990;36(11):1922-7.
25. Frosali S, Di Simplicio P, Perrone S, Di Giuseppe D, Longini M, Tanganelli D, et al. Glutathione recycling and antioxidant enzyme activities in erythrocytes of term and preterm newborns at birth. *Biol Neonate* 2004;85(3):188-94.
26. Thibeault DW. The precarious antioxidant defenses of the preterm infant. *American journal of perinatology* 2000;17(4):167-82.
27. Levonen AL, Lapatto R, Saksela M, Raivio KO. Expression of gamma-glutamylcysteine synthetase during development. *Pediatr Res* 2000;47(2):266-70.
28. Lestas AN, Rodeck CH. Normal glutathione content and some related enzyme activities in the fetal erythrocytes. *Br J Haematol* 1984;57(4):695-702.
29. Martin JA, Pereda J, Martinez-Lopez I, Escrig R, Miralles V, Pallardo FV, et al. Oxidative stress as a signal to up-regulate gamma-cystathionase in the fetal-to-neonatal transition in rats. *Cell Mol Biol (Noisy-legrand)* 2007;53 Suppl:OL1010-7.
30. Pallardo FV, Sastre J, Asensi M, Rodrigo F, Estrela JM, Vina J. Physiological changes in glutathione metabolism in foetal and newborn rat liver. *Biochem J* 1991;274 (Pt 3):891-3.
31. Vina J, Vento M, Garcia-Sala F, Puertes IR, Gasco E, Sastre J, et al. L-cysteine and glutathione metabolism are impaired in premature infants due to cystathionase deficiency. *Am J Clin Nutr* 1995;61(5): 1067-9.
32. Te Braake FW, Schierbeek H, Vermes A, Huijmans JG, van Goudoever JB. High-dose cysteine administration does not increase synthesis of the antioxidant glutathione preterm infants. *Pediatrics* 2009; 124(5):e978-84.
33. Riedijk MA, van Beek RH, Voortman G, de Bie HM, Dassel AC, van Goudoever JB. Cysteine: a conditionally essential amino acid in low-birth-weight preterm infants? *Am J Clin Nutr* 2007;86(4):1120-5.
34. Riedijk MA, Voortman G, van Beek RH, Baartmans MG, Wafelman LS, van Goudoever JB. Cyst(e)ine requirements in enterally fed very low birth weight preterm infants. *Pediatrics* 2008;121(3):e561-7.
35. Thomas B, Gruca LL, Bennett C, Parimi PS, Hanson RW, Kalhan SC. Metabolism of methionine in the newborn infant: response to the parenteral and enteral administration of nutrients. *Pediatr Res* 2008; 64(4):381-6.

Chapter 9

A multicomponent lipid emulsion containing a mixture of soybean oil, medium-chain triglycerides, olive oil and fish oil does not reduce oxidative stress in very low birth weight infants; a double-blind randomized controlled trial

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ABSTRACT

Introduction

Parenteral lipids are an integral part of nutrition of preterm infants, but have been associated with increased oxidative stress. In preterm infants, oxidative stress is associated with major neonatal morbidities such as bronchopulmonary dysplasia and periventricular leukomalacia. Traditional lipid emulsions are mainly manufactured from soybean oil and are rich in ω-6 polyunsaturated fatty acids (PUFAs), which are highly susceptible for lipid peroxidation resulting in increased oxidative stress. The objective of this study was to compare the effect of administration of a multicomponent lipid emulsion containing soybean oil, medium-chain triglycerides, olive oil and fish oil versus a traditional pure soybean oil emulsion on oxidative stress markers and glutathione (GSH) kinetics in very low birth weight (VLBW) infants in a double-blind randomized controlled trial.

Methods

VLBW infants (birth weight < 1500 g) were randomized to receiving one of the lipid emulsions from birth onwards. All infants received glucose and amino acids (2.4-3.6 g/(kg•d)) from birth onwards. On day 2, infants received a primed, continuous infusion of [U-^{13}C]glycine, a precursor for glutathione synthesis, to determine intra-erythrocyte glutathione synthesis rates. Oxidative stress markers were measured in urine. Data are presented as median (interquartile range).

Results

Twenty-four infants with a gestational age of 26⅐ (25⅔-28) weeks and birth weight of 783 (649-980) g were included. GSH concentration (1.8 (1.4-2.0) mmol/L), fractional synthesis rate (41 (38-52) %/d) and absolute synthesis rate (6.6 (5.5-9.3) mg/(kg•d)) were not different between the two lipid emulsion groups. Total isofurans was reduced upon administration of the multicomponent lipid emulsion while the other oxidative stress markers, including isoprostanes, were not different between the lipid emulsions.

Conclusions

The multicomponent lipid emulsion, containing a mixture of soybean oil, medium-chain triglycerides, olive oil, and fish oil does not result in reduced oxidative stress compared to a pure soybean oil emulsion.

INTRODUCTION

Preterm infants who received amino acids and energy following birth have shown improved nitrogen balance, higher synthesis rates of specific proteins and enhanced growth compared to infants receiving glucose only[1 2]. Most studies aimed at improving nutritional management in preterm infants have been focused on amino acids. Lipids are frequently not started directly following birth or are started in low amounts[3], although positive associations between energy intake and neurodevelopmental development have been described[4-6].

Traditional lipid emulsions are mainly manufactured from soybean oil and are very rich in ω-6 polyunsaturated fatty acids (PUFAs), which are highly susceptible to lipid peroxidation resulting in increased oxidative stress[7-9]. Due to an immature antioxidant defense system, preterm infants are unable to counteract increased production of reactive oxygen species (ROS)[10-13]. This results in increased oxidative stress which is considered to play a role in the pathogenesis of bronchopulmonary dysplasia, necrotizing enterocolitis, retinopathy of prematurity and periventricular leukomalacia[14].

In newer lipid emulsions, soybean oil is (partially) replaced by other lipid sources, such as coconut oil (providing medium-chain triglycerides), olive oil and/or fish oil. These newer lipid emulsions might positively influence oxidative stress by a more balanced ω-6 : ω-3 fatty acid ratio. Furthermore, the increased α-tocopherol content in newer lipid emulsions and the phenolic compounds present in olive oil are strong antioxidants[15]. Previously, it has been shown that a multicomponent lipid emulsion increased the total antioxidant potential (TAP) in preterm infants compared to a pure soybean oil emulsion[16]. However, other studies comparing an olive oil-based lipid emulsion or a multicomponent lipid emulsion with a pure soybean oil emulsion could not demonstrate such differences in oxidative stress[17-20].

Since the results are conflicting, we performed a double-blind, randomized study in very low birth weight (VLBW; birth weight <1500g) infants and hypothesized that administration of a multicomponent lipid emulsion, containing a more balanced ω-6 : ω-3 fatty acid ratio and added α-tocopherol, would result in reduced oxidative stress, measured via oxidative stress markers and glutathione (GSH) kinetics, compared to administration of a traditional pure soybean oil emulsion.

SUBJECTS AND METHODS

This double-blind, randomized study was performed at the Neonatal Intensive Care Unit of the Erasmus MC - Sophia Children's Hospital (Rotterdam, the Netherlands) between 2009 and 2012. The study protocol was approved by the institutional medical ethical review board.

Subjects

Inborn VLBW infants with a central venous and arterial catheter inserted for clinical purposes were eligible for the study. Exclusion criteria were congenital anomalies, including chromosome defects, and infants with metabolic diseases, or endocrine, renal or hepatic disorders. Written informed consent was obtained from all parents prior to the study.

Randomization and masking

Within 6 hours after birth, the attending physician included the infant by opening a sealed opaque randomization envelope. Envelopes were prepared by a research-pharmacist, who was not involved in clinical care, based on a computer-generated block randomization list with variable block sizes, provided by a statistician. Lipids were supplied individually for each included infant by the hospitals' pharmacy in unidentifiable syringes. Randomization to the lipid group remained blinded throughout the whole study and analyses.

Nutritional intervention

Infants were randomly assigned to either Intralipid 20% (Control group; Fresenius Kabi, Germany) or SMOFlipid 20% (Intervention group; Fresenius Kabi, Germany) from birth onwards with a starting dose of 2 g/(kg·d) and 3 g/(kg·d) on the following days. Composition of the two lipids is depicted in **Table 1**. The infants received glucose and amino acids [Primene 10%, Baxter; 2.4-3.6 g/(kg·d)] from birth onwards. Whenever possible, minimal enteral feeding was

Table 1: Composition of the lipid emulsions.

	Intralipid 20%	SMOFlipid 20%
Soybean oil (g/L)	200	60
Medium-chain triglycerides (g/L)	-	60
Olive oil (g/L)	-	50
Fish oil (g/L)	-	30
Egg phospholipids (g/L)	12	12
Major fatty acids (% by wt)		
16:0, Palmitic acid	11	9.2
18:0, Stearic acid	4	2.7
18:1ω-9, Oleic acid	21	27.8
18:2ω-6, Linoleic acid	53	18.7
18:3ω-3, α-Linolenic acid	8	2.4
20:4ω-6, Arachidonic acid	-	0.5
20:5ω-3, Eicosapentaenoic acid	-	2.4
22:6ω-3, Docosahexaenoic acid	-	2.2
α-Tocopherol (mg/L)	~38	~200
ω-6 : ω-3 Fatty acid ratio	7:1	2.5:1

initiated on the day of birth and advanced to full enteral nutrition in the succeeding days according to local protocol.

Glutathione kinetics

Tracer infusion protocol and blood sampling

[U-^{13}C]glycine (99% enriched; prepared according good clinical practice (GMP); sterility and pyrogenicity tested; Cambridge Isotope Laboratories, Andover, MA, USA) was dissolved in a 0.9% saline solution by the hospital pharmacist according to GMP guidelines. On the second postnatal day, a primed (20 µmol/kg) continuous (20 µmol/(kg•h)) infusion of [U-^{13}C]glycine was administered during 8 hours using a Perfusor fm infusion pump (B Braun Medical B.V., Oss, the Netherlands). Blood was sampled from an indwelling arterial catheter after 6, 7 and 8 hours and collected in EDTA containing microtainers. The blood samples were immediately put on melting ice and centrifuged at 3500 x g for 10 min. The plasma fraction was removed, and the lower layer (containing primarily erythrocytes) was reconstituted to its original volume with ice-cold distilled water and stored at -80°C until analysis. To calculate the fractional synthesis rates (FSR) and absolute synthesis rates (ASR), concentrations and enrichments of GSH and its precursor glycine were determined in the erythrocytes.

Mass spectrometric analysis of glutathione enrichment and concentration

Measurement of GSH concentration and enrichment was performed with a LC-Isolink interface coupled to a Delta XP isotope ratio mass spectrometer (LC-IRMS) (Thermo Fisher, Bremen, Germany) using a previously described method[21]. This highly sensitive method requires only a very small sample amount, and does not require derivatization of the sample.

Mass spectrometric analysis of glycine enrichment

The erythrocyte enrichment of [U-^{13}C]glycine was measured by gas chromatography-mass spectrometry (GCMS) as their ethyl chloroformate (ECF) ester derivatives, using a MSD 5975C Agilent GCMS (Agilent Technologies, Amstelveen, the Netherlands). Briefly, 25 µL aliquots of the remaining supernatant used for the GSH analysis were taken, acidified by adding 50 µL of 0.1 M HCl and diluted with 125 µL of distilled water. ECF derivatization of the samples was performed according to a modified procedure of Hušek[22]. A VF-17ms column (30 m x 0.25 mm id, 0.12 µm film thickness; Varian, Middelburg, the Netherlands) was used for the separation. The samples were measured using a selected ion monitoring (SIM) method in electron impact mode. The mass fragments with a mass to charge (m/z) of 102.1 for unenriched and a m/z 103.1 for the enriched glycine respectively, were selected for this purpose (in this fragment one ^{13}C atom was lost due to fragmentation in the source). Each sample was analyzed in triplicate by GCMS. Enrichments were calculated from the mean of the triplicate analyses and expressed as mole percent excess (MPE).

Calculations

The FSR_{GSH} represents the fraction of the total intraerythrocytic GSH pool that is renewed per unit of time, expressed as %/d.

$$FSR_{GSH}\,(\%/d) = \frac{\text{slope } E_{[U\text{-}^{13}C]GSH_{t6,7,8}}}{E_{\text{intraerythrocystic}[U\text{-}^{13}C]\text{glycine}}} \times 24h \times 100\%$$

where E stands for the enrichment expressed as MPE. The nominator (product) of this equation represents the hourly increase in [U-^{13}C]glycine bound GSH, as calculated from the increase in enrichment between 6 and 8 hours of infusion. The denominator (precursor) represents the intraerythrocytic [U-^{13}C] enrichment of free glycine at isotopic steady state. A steady-state plateau was defined as an insignificant change in intraerythrocytic enrichment over time. Subsequently, the intravascular ASR_{GSH}, which represents the absolute amount of GSH that is produced per unit of time (mg/(kg×d)), can be calculated using the following equation:

$$ASR_{GSH}\,(mg/(kg \bullet d)) = FSR_{GSH}/(100 \times conc. \times 307 \times ht \times 0.075),$$

where conc. is GSH concentration in mmol/L packed erythrocytes, 307 is the molecular weight of GSH, ht is hematocrit and 0.075 is the estimated circulating volume in a preterm neonate, expressed as L/kg.

Urinary oxidative stress markers

On postnatal day 2, 4 and 6, urine samples were collected. After centrifugation at 2800 x g for 5 minutes, the samples were stored at -20°C until analysis. Urine samples were shipped on dry ice to the University & Polytechnic Hospital La Fe (Valencia, Spain), where the urinary oxidative stress markers were determined.

Urinary 8-hydroxy-2'-deoxyguanosine/2-deoxyguanosine ratio (8OhdG/2dG; marker of oxidative damage to DNA), Ortho-tyrosine/phenylalanine ratio (O-Tyr/Phe; marker of oxidative damage to protein), 3-Nitro-tyrosine (3-N-Tyr; marker of nitrosative damage to protein) and 3-Chloro-tyrosine (3-Cl-Tyr; marker of inflammation caused by free radicals) were determined by ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), modified for 3-N-Tyr and 3-Cl-Tyr based on the method previously described by Orhan et al.[23 24]. UPLC-MS/MS was carried out at the Central Service for the Support to Experimental Research (SCSIE) of the University of Valencia, using an Acquity UPLC TQ Detector (Waters, Manchester, UK). Analytical separation was performed using a core shell C18 Acquity UPLC® BEH column (2.1x 50mm, 1.7 µm, Waters). Transitions (m/z) and retention times for each considered analyte were the following, Phe (166.1>120.1; 1.7 min), O-Tyr (182.05>136.1; 1.38 min), 3-N-Tyr (227.1>181.2; 1.96 min), 3-Cl-Tyr (216.1>170.1; 1.60 min), 8OhdG (284.1>168.1; 0.91 min) and 2dG (268.1>152.1; 0.92 min).

For urinary isoprostanes, isofurans (both derived from peroxidation of predominantly arachidonic acid, but also linolenic acid and eicosapentaenoic acid), neuroprostanes and neurofurans (both derived from peroxidation of docosahexaenoic acid) determinations, samples were subjected to a solid phase extraction procedure for clean-up and providing a pre-concentration factor of 10. If the sample volume was too low for the direct application of the procedure, samples were diluted in H_2O prior to the extraction procedure. Chromatographic separations were carried out using a Kinetex C18 column (2.1x100, 1.7 µM, Phenomenex, USA) running a linear CH_3OH:H_2O gradient from 30% to 90% CH_3OH in 4 min at a flow of 0.4 ml/min and using an injection volume of 5 µL. Chromatograms for total isoprostanes, isofurans, neuroprostanes and neurofurans were recorded as described previously[25]. Results were expressed as 'total response' because, due to the lack of commercially available standards, only relative response values were available.

Statistical analysis

Statistical analysis was performed using SPSS PASW 17 (SPSS Inc, Chicago, IL, USA). Unless otherwise stated, data are expressed as medians (interquartile range). The primary outcome of this study was the FSR_{GSH}. Power calculation showed that, in order to detect a difference in FSR_{GSH} of 10%/d and a SD of 6% (based on our earlier study[26]) with an α of 0.05 and a power of 0.80, group size needed to be 7 in each group. To allow for drop outs due to analytical problems and to ensure adequate numbers after de-blinding, we included 24 infants in total.

For continuous data, differences between groups were determined using the Mann-Whitney test and for categorical data, differences were determined using the Chi-Square test. A p value of <0.05 was considered as statistically significant.

RESULTS

The trial profile is depicted in **Figure 1**. Due to the study design, parental informed consent had to be obtained within 6 hours of birth, which was not possible in 55 infants. After randomization to the different nutritional regiments, not all infants were eligible for further analysis. The main reasons were reduction of nutrient administration according to clinical protocol (i.e. plasma urea concentration ≥ 10 mmol/L or triacylglycerol concentration ≥ 3 mmol/L) during the isotope study and clinical contra-indications for the isotope study (e.g., fluid restriction, restriction of blood withdrawals). In total, 24 VLBW infants were analyzed in this study.

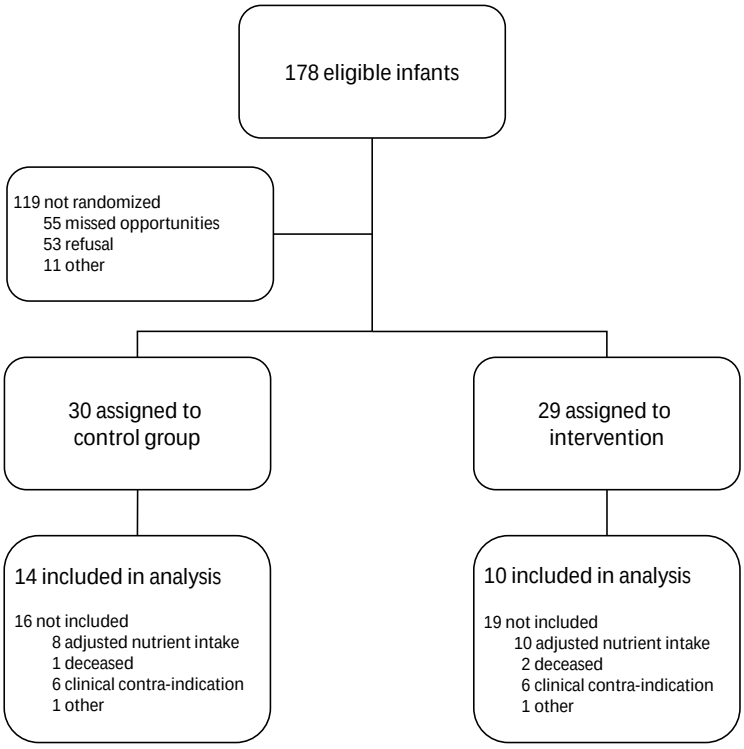

Figure 1: Trial profile.

Demographic and study characteristics

Patient characteristics are depicted in **Table 2**. Nutritional intake and oxygen administration on the study day (postnatal day 2) are displayed in **Table 3**.

Table 2: Baseline clinical characteristics of the infants included in the study.

	Control	Intervention
N (male:female)	14 (8:6)	10 (6:4)
Gestational age (weeks)	26⅚ (25⅔ - 28)	27⅓ (25 - 28⅙)
Birth weight (g)	783 (687 - 963)	790 (628 - 1000)
Birth weight Z-score	-1.2 (-2.6 - -0.6)	-1.9 (-2.5 - -0.5)
Cord blood pH	7.31 (7.28 - 7.34)	7.26 (7.18 - 7.32)
Apgar score at 5 min	8 (6 - 9)	8 (7 - 9)
CRIB score[§]	4 (1 - 8)	5 (2- 8)

Data are presented as median (interquartile range). [§]The CRIB score (Clinical Risk Index for Babies) indicates the degree of illness[27] . The score has a maximum of 23 points and is positively correlated with the severity of illness.

Table 3: Parenteral nutritional intake and oxygen administration on postnatal day 2.

		Control	Intervention
Amino acid intake in g/(kg×d)		3.3 ± 0.7	2.7 ± 0.6 *
Non-protein intake in kcal/(kg×d)		54 ± 10	54 ± 7
Total energy intake in kcal/(kg×d)		68 ± 11	65 ± 7
FiO_2 (%)	**Minimum**	21 (21 - 26)	21 (21 - 26)
	Maximum	35 (21 - 46)	33 (26 - 50)
	Median	24 (21 - 29)	24 (21 - 35)

Data are presented as mean ± SD or median (interquartile range). * $p < 0.05$.

GSH kinetics and oxidative stress markers

GSH kinetic data are presented in **Figure 2**. The concentrations of erythrocyte GSH, the FSR_{GSH} and the ASR_{GSH} were not different between groups.

Protein intake was higher in the control group, but this was not correlated with GSH concentration or synthesis (**Figure 3**). **Figure 4** depicts the urinary oxidative stress markers. Total isofurans were significantly decreased in the intervention group (day 2 p=0.009, day 4 p=0.003, and day 6 p=0.026). The other urinary markers of oxidative damage were not different between the groups.

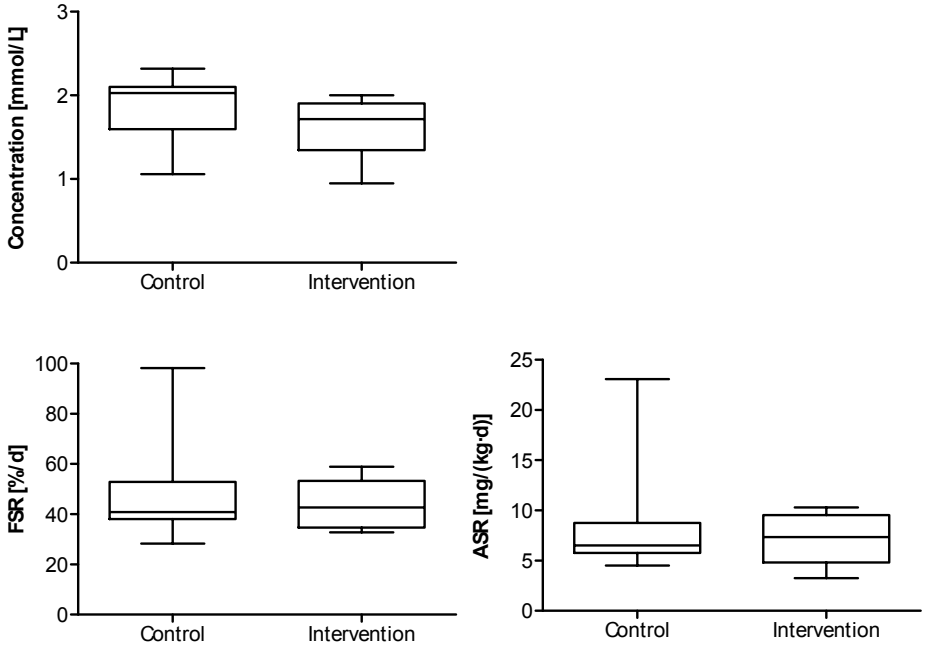

Figure 2: Glutathione synthesis rates and concentrations on the second day of life.
Data are shown as box-plots. Glutathione concentration, fractional synthesis rates (FSR) and absolute synthesis rates (ASR) were similar in the groups.

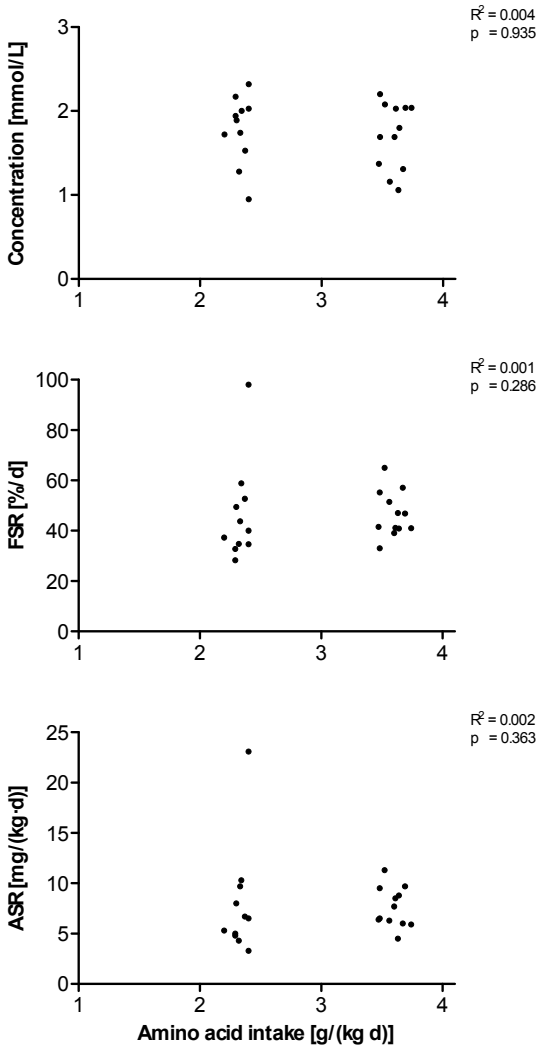

Figure 3: Correlation between amino acid intake and GSH synthesis on the second day of life.
Correlations were analyzed using Spearman's correlation coefficient. FSR = fractional synthesis rate, ASR = absolute synthesis rate.

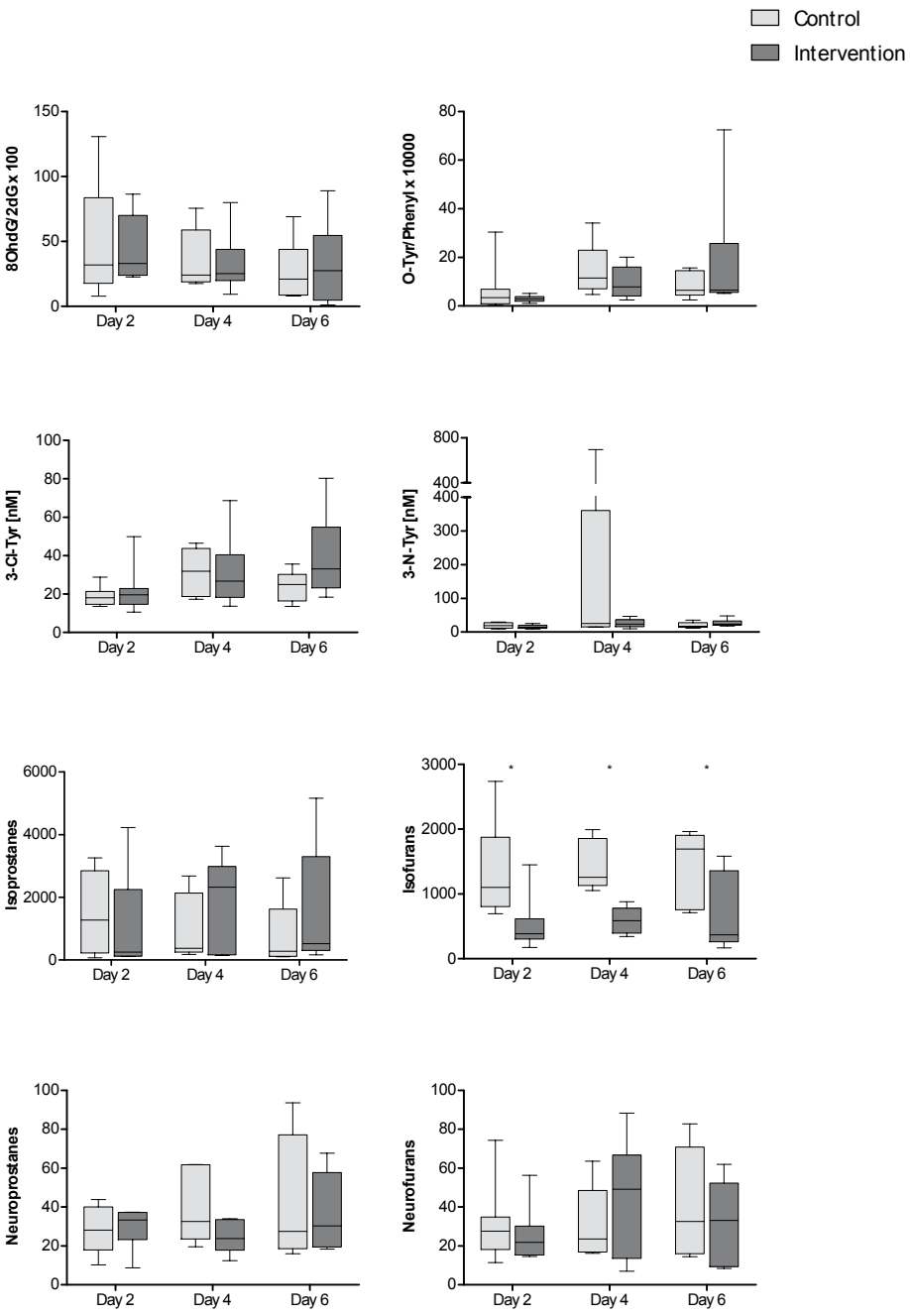

Figure 4: Urinary oxidative stress markers in the first week of life.
Data are shown as box-plots. 8OhdG/2dG = 8-hydroxy-2'-deoxyguanosine/2-deoxyguanosine ratio, O-Tyr/Phe = Ortho-tyrosine/phenylalanine ratio, 3-N-Tyr = 3-Nitro-tyrosine, 3-Cl-Tyr = 3-Chloro-tyrosine. Total isoprostanes, isofurans, neuroprostanes and neurofurans are expressed as 'total response'. * $p < 0.05$

DISCUSSION

In this study, we demonstrate that administration of a multicomponent lipid emulsion containing a mixture of soybean oil, medium-chain triglycerides, olive oil, and fish oil does not result in either altered GSH concentration or synthesis rates compared to administration of a pure soybean oil emulsion in VLBW infants. Besides a decrease in isofurans (indicative of a reduced arachidonic acid peroxidation), all other markers of oxidative stress were not reduced in infants receiving a multicomponent lipid emulsion. Taken together, there does not seem to be a clear reduction of oxidative stress upon administration of the multicomponent lipid emulsion compared to a pure soybean oil emulsion. As a consequence, GSH concentration and synthesis were not altered upon administration of the two lipids.

Lipid peroxidation is a self-propagating chain-reaction with the formation of lipid radicals, which will readily react with other lipid molecules. PUFAs are especially prone for lipid peroxidation, because they contain multiple double bonds with reactive hydrogens. Isoprostanes are prostaglandin-like compounds, which are formed from the peroxidation of mainly arachidonic acid, linolenic acid and eicosapentaenoic acid[28 29]. Peroxidation of docosahexaenoic acid results in the formation of neuroprostanes[29]. α-Tocopherol is an important scavenger of peroxyl radicals, which are intermediates in lipid peroxidation. By reacting with the peroxyl radicals, a tocopheryl radical is formed which will then be reduced by a hydrogen donor, such as vitamin C. The more balanced fatty acid composition and increased α-tocopherol content in the multicomponent lipid emulsion did, however, not result in decreased formation of isoprostanes and/or neuroprostanes.

There are several possible explanations for this finding. As depicted in **Table 1**, the content of linoleic acid is considerably lower in the multicomponent lipid emulsion, but content of arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid is slightly higher. The fatty acid composition of the multicomponent lipid emulsion might therefore still be pro-oxidant. Second, the levels of 8OhdG/2dG and o-Tyr/Phenyl found in our study are increased from day two onwards compared to levels found shortly after birth[30 31]. Oxidative stress is possibly already so high in preterm infants that parenteral lipids do not seem to contribute significantly to this oxidative burden. This is underscored by a recent meta-analyses showing no differences in incidence of major neonatal morbidities associated with oxidative stress in relation with the timing or type of lipid administration[32].

Administration of the multicomponent lipid emulsion was associated with a reduction of isofurans. Isofurans are similar to isoprostanes, but contain a substituted tetrahydrofuran ring. The formation of isofurans seems to be affected by oxygen tension; at elevated oxygen concentrations and otherwise similar conditions, the formation of isofurans is favored whereas the formation of isoprostanes is unaffected[29]. The discrepancy between the reduced isofurans while isoprostanes were unaffected upon administration of a multicomponent lipid emulsion suggests an interplay between lipid peroxidation and oxygen, rather than an effect of sole lipid

peroxidation. This is in agreement with previous studies comparing an olive oil based lipid emulsion or a multicomponent lipid emulsion with a pure soybean oil emulsion, demonstrating no differences in oxidative stress[17-20].

The main strength of the present study is the double-blind, randomized study design. Furthermore, in our study, lipids were initiated with a starting dose of 2 g/(kg·d) directly from birth onwards, while in previous studies parenteral lipids were supplied either in low doses from birth onwards or lipid administration was not started directly from birth onwards. Also, we measured both the synthesis of the main intracellular antioxidant and multiple markers of oxidative damage. Especially urinary isoprostanes are a well-known marker of lipid peroxidation[33 34]. A possible limitation is the exclusion of infants in whom nutrition was reduced based on plasma urea or triglyceride concentration. These infants might be the smallest and sickest infants creating a selection bias. However, this was the case in both study groups and the decision to exclude these infants was made to ensure comparison of oxidative stress at intended nutritional intake.

In conclusion, a lipid emulsion containing a mixture of soybean oil, medium-chain triglycerides, olive oil, and fish oil does not result in reduced oxidative stress compared to a traditional pure soybean oil emulsion in preterm infants. However, caution is needed in interpreting these results. Although the association of a reduction in isofurans seems to suggest an interplay between lipid peroxidation and oxygen rather than an effect of sole lipid peroxidation, the other markers were not affected and the type of lipid does not affect neonatal morbidities[32], the implication of differences in isofurans is unknown.

REFERENCES

1. van den Akker CH, Vlaardingerbroek H, van Goudoever JB. Nutritional support for extremely low-birth weight infants: abandoning catabolism in the neonatal intensive care unit. *Curr Opin Clin Nutr Metab Care* 2010;13(3):327-35.
2. Vlaardingerbroek H, van Goudoever JB, van den Akker CH. Initial nutritional management of the preterm infant. *Early Hum Dev* 2009;85(11):691-5.
3. Lapillonne A, Fellous L, Kermorvant-Duchemin E. Use of parenteral lipid emulsions in French neonatal ICUs. *Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition* 2011;26(6):672-80.
4. Ehrenkranz RA, Das A, Wrage LA, Poindexter BB, Higgins RD, Stoll BJ, et al. Early nutrition mediates the influence of severity of illness on extremely LBW infants. *Pediatr Res* 2011;69(6):522-9.
5. Eleni Dit Trolli S, Kermorvant-Duchemin E, Huon C, Bremond-Gignac D, Lapillonne A. Early lipid supply and neurological development at one year in very low birth weight (VLBW) preterm infants. *Early Hum Dev* 2012;88 Suppl 1:S25-9.
6. Stephens BE, Walden RV, Gargus RA, Tucker R, McKinley L, Mance M, et al. First-week protein and energy intakes are associated with 18-month developmental outcomes in extremely low birth weight infants. *Pediatrics* 2009;123(5):1337-43.
7. Helbock HJ, Motchnik PA, Ames BN. Toxic hydroperoxides in intravenous lipid emulsions used in preterm infants. *Pediatrics* 1993;91(1):83-7.
8. Laborie S, Lavoie JC, Chessex P. Increased urinary peroxides in newborn infants receiving parenteral nutrition exposed to light. *J Pediatr* 2000;136(5):628-32.
9. Alexander-North LS, North JA, Kiminyo KP, Buettner GR, Spector AA. Polyunsaturated fatty acids increase lipid radical formation induced by oxidant stress in endothelial cells. *Journal of lipid research* 1994;35(10):1773-85.
10. Georgeson GD, Szony BJ, Streitman K, Varga IS, Kovacs A, Kovacs L, et al. Antioxidant enzyme activities are decreased in preterm infants and in neonates born via caesarean section. *Eur J Obstet Gynecol Reprod Biol* 2002;103(2):136-9.
11. Buhimschi IA, Buhimschi CS, Pupkin M, Weiner CP. Beneficial impact of term labor: nonenzymatic antioxidant reserve in the human fetus. *Am J Obstet Gynecol* 2003;189(1):181-8.
12. Comporti M, Signorini C, Leoncini S, Buonocore G, Rossi V, Ciccoli L. Plasma F2-isoprostanes are elevated in newborns and inversely correlated to gestational age. *Free radical biology & medicine* 2004;37(5):724-32.
13. Buonocore G, Perrone S, Longini M, Vezzosi P, Marzocchi B, Paffetti P, et al. Oxidative stress in preterm neonates at birth and on the seventh day of life. *Pediatr Res* 2002;52(1):46-9.
14. Saugstad OD. The oxygen radical disease in neonatology. *Indian J Pediatr* 1989;56(5):585-93.
15. Tuck KL, Hayball PJ. Major phenolic compounds in olive oil: metabolism and health effects. *The Journal of nutritional biochemistry* 2002;13(11):636-44.
16. Skouroliakou M, Konstantinou D, Koutri K, Kakavelaki C, Stathopoulou M, Antoniadi M, et al. A double-blind, randomized clinical trial of the effect of omega-3 fatty acids on the oxidative stress of preterm neonates fed through parenteral nutrition. *Eur J Clin Nutr* 2010;64(9):940-7.
17. Koksal N, Kavurt AV, Cetinkaya M, Ozarda Y, Ozkan H. Comparison of lipid emulsions on antioxidant capacity in preterm infants receiving parenteral nutrition. *Pediatr Int* 2011;53(4):562-6.
18. Roggero P, Mosca F, Gianni ML, Orsi A, Amato O, Migliorisi E, et al. F2-isoprostanes and total radical-trapping antioxidant potential in preterm infants receiving parenteral lipid emulsions. *Nutrition* 2009.
19. Gobel Y, Koletzko B, Bohles HJ, Engelsberger I, Forget D, Le Brun A, et al. Parenteral fat emulsions based on olive and soybean oils: a randomized clinical trial in preterm infants. *Journal of pediatric gastroenterology and nutrition* 2003;37(2):161-7.
20. Webb AN, Hardy P, Peterkin M, Lee O, Shalley H, Croft KD, et al. Tolerability and safety of olive oil-based lipid emulsion in critically ill neonates: a blinded randomized trial. *Nutrition* 2008;24(11-12):1057-64.

21. Schierbeek H, Te Braake F, Godin JP, Fay LB, van Goudoever JB. Novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2007;21(17):2805-12.
22. Hušek P. Rapid derivatization and gas chromatographic determination of amino acids. *Journal of Chromatography A* 1991;552(0):289-99.
23. Orhan H, Vermeulen NP, Tump C, Zappey H, Meerman JH. Simultaneous determination of tyrosine, phenylalanine and deoxyguanosine oxidation products by liquid chromatography-tandem mass spectrometry as non-invasive biomarkers for oxidative damage. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004;799(2):245-54.
24. Solberg R, Andresen JH, Escrig R, Vento M, Saugstad OD. Resuscitation of hypoxic newborn piglets with oxygen induces a dose-dependent increase in markers of oxidation. *Pediatr Res* 2007;62(5):559-63.
25. Song WL, Lawson JA, Reilly D, Rokach J, Chang CT, Giasson B, et al. Neurofurans, novel indices of oxidant stress derived from docosahexaenoic acid. *The Journal of biological chemistry* 2008;283(1): 6-16.
26. Te Braake FW, Schierbeek H, de Groof K, Vermes A, Longini M, Buonocore G, et al. Glutathione synthesis rates after amino acid administration directly after birth in preterm infants. *Am J Clin Nutr* 2008; 88(2):333-9.
27. The CRIB (clinical risk index for babies) score: a tool for assessing initial neonatal risk and comparing performance of neonatal intensive care units. The International Neonatal Network. *Lancet* 1993; 342(8865):193-8.
28. Belik J, Gonzalez Luis GE, Perez Vizcaino F, Villamor E. Isoprostanes in fetal and neonatal health and disease. *Free radical biology & medicine* 2009;48(2):177-88.
29. Roberts LJ, 2nd, Fessel JP, Davies SS. The biochemistry of the isoprostane, neuroprostane, and isofuran Pathways of lipid peroxidation. *Brain Pathol* 2005;15(2):143-8.
30. Vento M, Aguar M, Escobar JJ, Arduini A, Escrig R, Brugada M, et al. Antenatal Steroids and Antioxidant Enzyme Activity in Preterm Infants: Influence of Gender and Timing. *Antioxid Redox Signal* 2009.
31. Vento M, Moro M, Escrig R, Arruza L, Villar G, Izquierdo I, et al. Preterm Resuscitation With Low Oxygen Causes Less Oxidative Stress, Inflammation, and Chronic Lung Disease. *Pediatrics* 2009;124(3):e439-49.
32. Vlaardingerbroek H, Veldhorst MA, Spronk S, van den Akker CH, van Goudoever JB. Parenteral lipid administration to very-low-birth-weight infants--early introduction of lipids and use of new lipid emulsions: a systematic review and meta-analysis. *Am J Clin Nutr* 2012.
33. Meagher EA, FitzGerald GA. Indices of lipid peroxidation in vivo: strengths and limitations. *Free radical biology & medicine* 2000;28(12):1745-50.
34. Milne GL, Sanchez SC, Musiek ES, Morrow JD. Quantification of F2-isoprostanes as a biomarker of oxidative stress. *Nat Protoc* 2007;2(1):221-6.

Part IV

Other modulators of oxidative stress

Chapter 10

Glutathione kinetics and oxidative stress markers in preterm infants with nosocomial sepsis

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ABSTRACT

Introduction

Preterm infants with sepsis have poorer long-term outcome than preterm infants without sepsis. In adults, increased free radical production during sepsis is associated with higher morbidity and mortality. Since antioxidant defense is reduced in preterm infants, we hypothesized that preterm infants are unable to upregulate synthesis of glutathione (GSH) - the major intracellular antioxidant - during sepsis, which may contribute to the detrimental long-term effects of sepsis.

Methods

A stable isotope study was performed in preterm infants with nosocomial sepsis (n=8) and preterm controls (n=6) to determine GSH kinetics and oxidative stress markers were measured in plasma.

Results

Gestational age (26⅝ (25⅐-28⅐) weeks) and birth weight (860 (610-1310) g) were not different between groups. The GSH fractional synthesis rate (45 (40-52) %/d), GSH absolute synthesis rate (11 (2.3-14) mg/(kg·d)), GSH concentration (2.6 (1.7-3.7) mmol/L) and non protein bound iron concentration (0.8 (0-5.8) μM) were not different between groups.

Conclusions

Non protein bound iron, GSH concentration and GSH synthesis were not altered, suggesting that oxidative stress is not increased during nosocomial sepsis in preterm infants. These results, albeit from a small number of patients, suggest that the detrimental outcome following nosocomial sepsis in premature infants seems not to be caused by increased oxidative stress.

INTRODUCTION

Despite major improvements in neonatal care, sepsis remains an important cause of morbidity and mortality in preterm infants. Reported incidence varies between 21 and 36%[1-3]. In a large survey, including 6956 preterm infants, those who developed late-onset sepsis were significantly more likely to die than those who did not (18% vs. 7%)[1]. Additionally, preterm infants who had an episode of late-onset sepsis during the first weeks were at increased risk of developing serious neonatal diseases such as patent ductus arteriosus, bronchopulmonary dysplasia and necrotizing enterocolitis[1]. Sepsis in preterm infants is also associated with severe adverse neurodevelopmental outcome at 2 years of age[4 5].

The relation between neonatal sepsis and the adverse neurodevelopmental outcome is likely to be multifactorial, but oxidative stress is thought to be one of the major contributors[4 6]. During sepsis, ROS formation is increased as part of the defensive response against pathogens through the stimulation of pro-inflammatory cytokine release, the formation of chemotactic factors and neutrophil recruitment[7-10]. In healthy adults, lipopolysaccharide stimulation results in an upregulation of glutathione (GSH) synthesis, the main intracellular antioxidant[11]. In both adults and children, overstimulation of ROS production or an insufficient antioxidant capacity is associated with increased morbidity and mortality[12-14]. In a study by Kapoor et al., non-survivors of neonatal sepsis had elevated levels of malondialdehyde, superoxide dismutase, glutathione peroxidase and catalase compared with infants who survived after neonatal sepsis[15]. A compromised antioxidant defense makes preterm infants more susceptible to oxidative stress[16 17], and the increased ROS formation during sepsis might contribute to the detrimental long-term outcome in preterm infants after neonatal sepsis.

In this study, we hypothesized that nosocomial sepsis in preterm infants results in increased oxidative stress, but without a concomitant upregulation of GSH synthesis. To test this hypothesis, we determined GSH synthesis and oxidative stress markers in preterm infants with nosocomial sepsis and in controls.

METHODS

Subjects

Preterm infants with a birth weight <1500 g admitted to the Neonatal Intensive Care Unit (NICU) of the Erasmus MC-Sophia Children's Hospital (Rotterdam, The Netherlands) were eligible for inclusion. Exclusion criteria were known congenital abnormalities or chromosomal defects; metabolic, endocrine, renal or hepatic disorders; and erythrocyte transfusions during the study.

Parental informed consent was obtained during the first days after birth. If infants were clinically suspected of having nosocomial sepsis (>72 hours of postnatal age) and antimicrobial treatment was initiated, they were studied within 24 hours. Based on laboratory data and clinical

symptoms, the diagnosis of nosocomial sepsis was confirmed or rejected by the attending physician after 48 hours, with subsequent continuation or discontinuation of therapy. Patients were included in the sepsis group if they met the Centers for Disease Control and Prevention criteria for nosocomial infections with the adjusted clinical signs for the NICU population by van der Zwet et al.[3 18]. Infants who were not diagnosed with nosocomial sepsis served as the control group.

Tracer infusion protocol

Infants received a primed (40 µmol/kg), continuous (20 µmol/(kg•h)) infusion of [1-^{13}C]glycine or [U-^{13}C]glycine for a period of 8 hours within 24 hours after the start of antimicrobial treatment. The [1-^{13}C]glycine and [U-^{13}C]glycine (99% enriched, sterility and pyrogenicity tested) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and were diluted in a 0.9% saline solution by the hospital pharmacy, after which they were again tested for sterility and pyrogenicity. Tracers were infused using a Perfusor fm infusion pump (B|Braun Medical B.V., Oss, The Netherlands) along the same route as the parenterally administered nutrition. Blood was sampled from an indwelling arterial catheter after 6, 7 and 8 hours in heparin-containing microtainers. If an arterial catheter was not present, blood was sampled via heel puncture after 6 and 8 hours, combined with routine blood withdrawal if possible. GSH concentration and synthesis rates were quantified in erythrocytes.

Sample analyses

Blood samples were immediately put on melting ice and subsequently centrifuged at 3500 x *g* for 10 min at 4°C. The plasma fraction was removed and stored separately for measurement of oxidative stress markers. The lower layer, containing primarily erythrocytes, was reconstituted to its original volume with ice-cold distilled water. The plasma fraction and the cell fraction were subsequently stored at -80°C until further analysis.

Glutathione enrichments and concentration

To calculate FSR and ASR, enrichments of GSH and its precursor glycine as well as GSH concentrations were determined. For this purpose, a recently described technique based on an LC-Isolink interface (Thermo Electron, Bremen, Germany) coupled to a Delta XP isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany) (LC-IRMS) was used[19]. This highly sensitive method requires a very small sample volume and does not require derivatization of the sample. The concentration of GSH in erythrocytes was measured by LC-IRMS using an internal standard method described previously[19].

Calculations

The FSR_{GSH} represents the fraction of the total intraerythrocytic GSH pool that is renewed per unit of time and is expressed as %/d.

$$FSR_{GSH}\ (\%/d) = \frac{\text{slope } E_{[1\text{-}^{13}C]GSH_{t4,5,6}}}{E_{\text{intraerythrocystic}[1\text{-}^{13}C]\text{glycine}}} \times 24h \times 100\%$$

In the equation, E stands for enrichment, which is expressed as mole percent excess (MPE). The nominator (product) of this equation represents the hourly increase in [1-^{13}C]glycine bound GSH calculated from the increase in enrichment between 6 and 8 hours of infusion. The denominator (precursor) represents the intraerythrocytic 1-^{13}C enrichment of free glycine at isotopic steady state. In subjects in whom [U-^{13}C]glycine was used, the [1-^{13}C]glycine in the equation was substituted with [U-^{13}C]glycine.

Subsequently, the intravascular ASR_{GSH}, which represents the absolute amount of GSH that is produced per unit of time (mg/(kg·d)), can be calculated using the following equation:

$$ASR_{GSH}\ (mg/(kg{\cdot}d) = FSR_{GSH}/(100 \times conc. \times 307 \times ht \times 0.075)$$

where conc. is GSH concentration in mmol/L packed erythrocytes, 307 is the molecular weight of GSH, ht is hematocrit, and 0.075 is the estimated circulating volume in a preterm infant, expressed as L/kg.

Plasma markers of oxidative stress

Plasma samples were shipped to the University of Siena (Italy), where the concentrations of isoprostane, a marker for lipid peroxidation, and non-protein bound iron (NPBI), an indirect marker of increased free radical release, were determined according to methods described previously[20 21].

RESULTS

In total, 12 preterm infants who were clinically suspected of having nosocomial sepsis were included and studied within 24 hours after antimicrobial treatment was instituted. In retrospect, 7 infants were classified as having blood culture proven sepsis or clinical sepsis and 5 as controls. In order to include more controls, 2 preterm infants without any suspicion of sepsis were included and studied on day 6 of life.

Patient characteristics at baseline and clinical characteristics during the study are displayed in **Table 1**. According to the study design, the highest measured C-reactive protein (CRP) and the number of positive blood cultures were different between the groups.

Table 1: Patient characteristics at baseline and clinical characteristics during the study.

	Control	Sepsis
N (male:female)	6 (2:4)	8 (7:1) *
Gestational age (weeks)	27⅐ (26⅐ – 28⅐)	26⅐ (25 - 29⅐)
Birth weight (g)	798 (693-1055)	898 (705-1025)
CRIB score[§]	4 (1-9)	6 (3-8)
Postnatal age during study (days)	6 (3-10)	11 (7-14)
CRP (mg/l)	2 (1-2)	59 (15-88) *
Positive blood culture	0/6	4/8 *
Median FiO_2 (%)	25 (21-29)	34 (23-40)
Nutritional intake		
Protein intake (g/(kg·d))	2.2 (1.7 - 2.7)	2.5 (2.1 - 3.0)
Total energy intake (kcal/(kg·d))	94 (90-102)	90 (74-98)

Values are presented as medians (interquartile range). * $p < 0.05$. [§]The CRIB score (Clinical Risk Index for Babies) indicates the degree of illness[22]. The score has a maximum of 23 points and is positively correlated with the severity of illness during the first 12 hours after birth. CRP = C-reactive protein. FiO_2 = fraction of inspired oxygen.

GSH concentrations and synthesis rates

Free intraerythrocytic [U-^{13}C]glycine enrichments reached a plateau after 4 - 6 hours of infusion. Enrichments did not differ between the sepsis group (median 2.8 (IQR 2.5-3.5)) and the control group (median 2.9 (IQR 2.7-3.2)). GSH kinetic data are presented in **Figure 1**. The concentration of erythrocyte GSH, the FSR_{GSH} and the ASR_{GSH} were not different between groups.

Oxidative stress markers

Due to the relative high volume of plasma needed for isoprostane determination, analysis could only be performed in 5 infants in each group. Plasma levels of isoprostanes and NPBI are presented in **Figure 2**. No differences were found between groups.

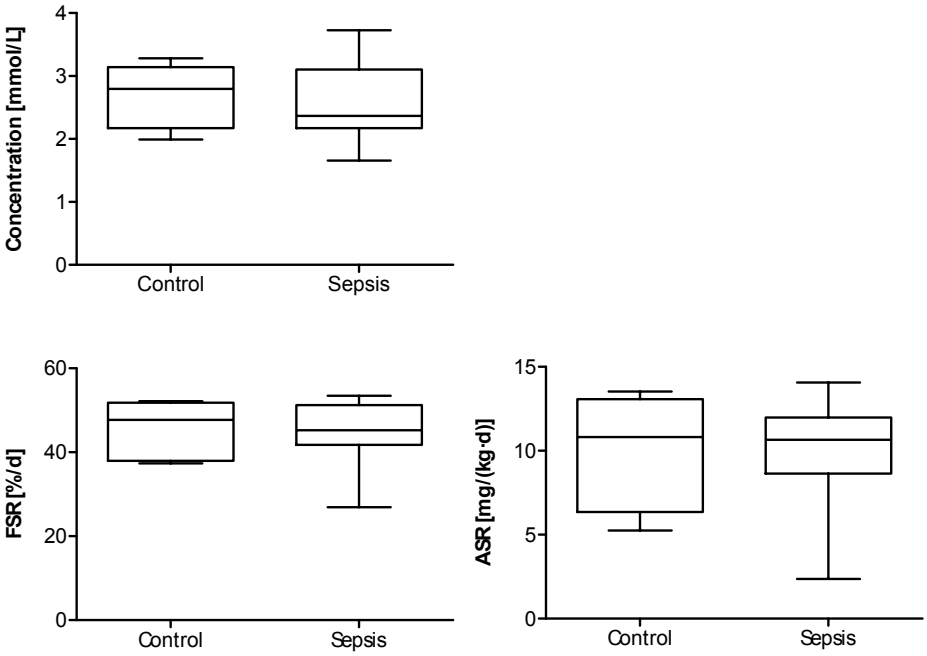

Figure 1: Glutathione concentrations, fractional synthesis rates and absolute synthesis rates.
Data are shown as box-plots. Glutathione concentration, fractional synthesis rate (FSR) and absolute synthesis rate (ASR) were similar in the groups.

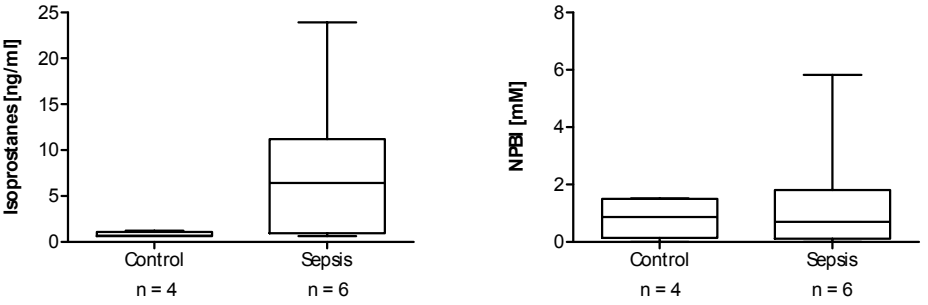

Figure 2: Plasma concentrations of isoprostanes and non protein bound iron.
Data are shown as box-plots. Isoprostanes and non protein bound iron (NPBI) were not different between the groups.

DISCUSSION

Nosocomial sepsis in premature infants does not result in decreased GSH concentrations or increased NPBI levels. There might be an increase in isoprostane levels, but the sample size was too small to detect statistically significant differences. The lack of difference in GSH concentrations could be explained by an upregulation of GSH synthesis rates, but we show in this study that neither the fractional nor the absolute GSH synthesis rate was upregulated. Therefore, these data suggest that nosocomial sepsis in preterm infants does not result in increased oxidative stress.

In preterm infants, sepsis is associated with increased morbidity and mortality, including adverse neurodevelopmental outcome later in life[1 4 5]. One possible mechanism for this association might be increased oxidative stress during sepsis[4 6]. Preterm infants have reduced antioxidant defenses and may be unable to counteract the increased ROS production during sepsis, resulting in increased oxidative stress. Previous studies demonstrated increased concentration of malondialdehyde and increased activity of certain antioxidant enzymes in, mostly term, neonates[15 23]. In this study, we were not able to demonstrate increased oxidative stress in preterm infants during sepsis.

The lack of differences between the groups could be explained by the design of our study, which resulted in a suboptimal control group. The control group mainly consisted of preterm infants suspected of having sepsis, but who, in retrospect, did not have confirmed sepsis because they did not fulfill the generally accepted criteria for sepsis[3]. It could be possible that they had an underlying condition that made physicians suspect sepsis such as respiratory or circulatory failure, which also caused an increase in oxidative stress. However, we found it unethical to draw blood samples from otherwise healthy preterm infants. In addition, using a different definition of late-onset sepsis in preterm infants, namely the definition used by the National Institute of Child Health and Human Development (NICHD) Neonatal Research Network[1], identical groups would have been formed.

The infants with nosocomial sepsis showed higher levels of isoprostanes (median 6.4 (0.96-11.2) ng/ml) compared with levels found directly after birth (0.13 ± 0.004 ng/ml), whereas the levels in the control group were closer to the levels found in newborns[24]. NPBI levels in both groups were within the range found in umbilical samples at birth of preterm infants[25]. However, we want to emphasize that the study was not powered to detect differences in concentration of oxidative stress markers.

In all patients, nosocomial sepsis was caused by coagulase-negative streptococci, the most common causative of nosocomial infection in preterm infants[1]. These infections are known to have a mild onset and are much less severe than septicemia caused by, for example, gram-negative bacteria. This might explain the lack of response in either GSH concentration or NPBI. The effect of a septic insult on GSH concentration and synthesis rates was apparent in healthy volunteers, demonstrating that GSH responds to sepsis[11]. However, GSH synthesis might be

limited by substrate availability in sick preterm infants, resulting in the absence of GSH upregulation in response to sepsis[26].

In conclusion, no differences in GSH synthesis, GSH concentration or oxidative stress markers were found between the preterm infants with nosocomial sepsis and the controls. These results suggest that oxidative stress in preterm infants is already high during the first weeks of life and that nosocomial sepsis does not cause an additional increase. Our data do not explain the association between nosocomial sepsis in preterm infants and adverse long-term outcome. Further research into other mechanisms, such as the production of pro-inflammatory cytokines that are known to be neurotoxic and altered cerebral blood flow through circulatory insufficiency, is warranted.

REFERENCES

1. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, et al. Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. *Pediatrics* 2002; 110(2 Pt 1):285-91.
2. Stoll BJ, Hansen NI, Bell EF, Shankaran S, Laptook AR, Walsh MC, et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics* 2010;126(3):443-56.
3. van der Zwet WC, Kaiser AM, van Elburg RM, Berkhof J, Fetter WP, Parlevliet GA, et al. Nosocomial infections in a Dutch neonatal intensive care unit: surveillance study with definitions for infection specifically adapted for neonates. *J Hosp Infect* 2005;61(4):300-11.
4. Adams-Chapman I, Stoll BJ. Neonatal infection and long-term neurodevelopmental outcome in the preterm infant. *Current opinion in infectious diseases* 2006;19(3):290-7.
5. Stoll BJ, Hansen NI, Adams-Chapman I, Fanaroff AA, Hintz SR, Vohr B, et al. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. *Jama* 2004; 292(19):2357-65.
6. Volpe JJ. Neurobiology of periventricular leukomalacia in the premature infant. *Pediatr Res* 2001; 50(5):553-62.
7. Martins PS, Kallas EG, Neto MC, Dalboni MA, Blecher S, Salomao R. Upregulation of reactive oxygen species generation and phagocytosis, and increased apoptosis in human neutrophils during severe sepsis and septic shock. *Shock* 2003;20(3):208-12.
8. de Souza LF, Ritter C, Pens Gelain D, Andrades M, Bernard EA, Moreira JC, et al. Mitochondrial superoxide production is related to the control of cytokine release from peritoneal macrophage after antioxidant treatment in septic rats. *J Surg Res* 2007;141(2):252-6.
9. Hansson L, Seidegard J, Johansson L, Jeppsson B. Influence of glutathione metabolising enzymes in rats with gram-negative sepsis. *Eur J Surg* 2000;166(9):728-33.
10. Andrades M, Ritter C, Moreira JC, Dal-Pizzol F. Oxidative parameters differences during non-lethal and lethal sepsis development. *J Surg Res* 2005;125(1):68-72.
11. van der Crabben SN, Stegenga ME, Blumer RM, Ackermans MT, Endert E, Tanck MW, et al. Erythrocyte glutathione concentration and production during hyperinsulinemia, hyperglycemia, and endotoxemia in healthy humans. *Metabolism* 2010.
12. Victor VM, Rocha M, De la Fuente M. Immune cells: free radicals and antioxidants in sepsis. *Int Immunopharmacol* 2004;4(3):327-47.
13. Crimi E, Sica V, Slutsky AS, Zhang H, Williams-Ignarro S, Ignarro LJ, et al. Role of oxidative stress in experimental sepsis and multisystem organ dysfunction. *Free Radic Res* 2006;40(7):665-72.
14. von Dessauer B, Bongain J, Molina V, Quilodran J, Castillo R, Rodrigo R. Oxidative stress as a novel target in pediatric sepsis management. *J Crit Care* 2010.
15. Kapoor K, Basu S, Das BK, Bhatia BD. Lipid peroxidation and antioxidants in neonatal septicemia. *J Trop Pediatr* 2006;52(5):372-5.
16. Ahola T, Levonen AL, Fellman V, Lapatto R. Thiol metabolism in preterm infants during the first week of life. *Scand J Clin Lab Invest* 2004;64(7):649-58.
17. Davis JM, Auten RL. Maturation of the antioxidant system and the effects on preterm birth. *Semin Fetal Neonatal Med* 2010.
18. Garner JS, Jarvis WR, Emori TG, Horan TC, Hughes JM. CDC definitions for nosocomial infections, 1988. *Am J Infect Control* 1988;16(3):128-40.
19. Schierbeek H, Te Braake F, Godin JP, Fay LB, van Goudoever JB. Novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2007;21(17):2805-12.
20. Milne GL, Sanchez SC, Musiek ES, Morrow JD. Quantification of F2-isoprostanes as a biomarker of oxidative stress. *Nat Protoc* 2007;2(1):221-6.

21. Paffetti P, Perrone S, Longini M, Ferrari A, Tanganelli D, Marzocchi B, et al. Non-protein-bound iron detection in small samples of biological fluids and tissues. *Biol Trace Elem Res* 2006;112(3):221-32.
22. The CRIB (clinical risk index for babies) score: a tool for assessing initial neonatal risk and comparing performance of neonatal intensive care units. The International Neonatal Network. *Lancet* 1993; 342(8865):193-8.
23. Batra S, Kumar R, Seema, Kapoor AK, Ray G. Alterations in antioxidant status during neonatal sepsis. *Ann Trop Paediatr* 2000;20(1):27-33.
24. Comporti M, Signorini C, Leoncini S, Buonocore G, Rossi V, Ciccoli L. Plasma F2-isoprostanes are elevated in newborns and inversely correlated to gestational age. *Free radical biology & medicine* 2004;37(5):724-32.
25. Perrone S, Tataranno ML, Negro S, Longini M, Marzocchi B, Proietti F, et al. Early identification of the risk for free radical-related diseases in preterm newborns. *Early Hum Dev* 2010;86(4):241-4.
26. Te Braake FW, Schierbeek H, de Groof K, Vermes A, Longini M, Buonocore G, et al. Glutathione synthesis rates after amino acid administration directly after birth in preterm infants. *Am J Clin Nutr* 2008; 88(2):333-9.

Chapter 11

The relation between gender, gestational age and birth weight with GSH kinetics in preterm infants

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In progress

ABSTRACT

Background

Gender, gestational age (GA) and birth weight (BW) are important prognostic factors for long-term outcome of preterm infants. Since oxidative stress is associated with both morbidity and mortality in preterm infants, we hypothesized that gender, GA and BW would affect both glutathione (GSH) synthesis and GSH-GSSH ratio. The latter is an indicator for recycling of GSH, i.e. oxidation to its dimeric form GSSG and reduction back to GSH.

Methods

For the correlation with GSH synthesis rates, very low birth weight (VLBW) infants (BW < 1500 g) were included, who participated in several intervention studies aimed at improving glutathione kinetics. On day 2, all infants received a primed, continuous infusion of [1-^{13}C] or [U-^{13}C]glycine, a precursor for GSH synthesis, to determine GSH fractional synthesis rate (FSR). Absolute GSH synthesis rate (ASR) was calculated from the FSR and erythrocyte GSH concentration. In addition, GSH-GSSG ratio was measured in preterm infants with GA < 32 weeks on the first day of life in blood using a mass spectrometry technique.

Results

Gender, GA and BW were not correlated with total GSH concentration or synthesis rates (n = 109). GA and BW were also not correlated with GSH-GSSG ratio, but female gender was associated with decreased GSH-GSSG ratio ($p = 0.024$, n = 75).

Conclusion

Glutathione synthesis and recycling is not influenced by birth weight or gestational age in preterm infants. However, GSH-GSSG ratio was slightly reduced in female infants. The implication of this finding must be further investigated in a larger study measuring multiple oxidative stress biomarkers simultaneously.

INTRODUCTION

Preterm infants are highly susceptible for oxidative stress. Due to an immature antioxidant defense system[1 2], preterm infants are unable to counteract the increased production of reactive oxygen species (ROS) associated with amongst others supplemental oxygen administration and infections[3 4]. This results in increased oxidative stress which is associated with serious neonatal diseases like bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), retinopathy of prematurity (ROP), intraventricular hemorrhage (IVH) and periventricular leucomalacia (PVL)[5].

Gestational age (GA), birth weight (BW) and gender are important determinants of outcome after preterm birth as a shorter gestation, lower birth weight and male gender are associated with poorer outcome[6-9]. This effect might be partly mediated by differences in antioxidant capacity. The antioxidant enzyme system is upregulated during the last 15% of gestation, a timeframe when non-enzymatic antioxidants are also crossing the placenta in increasing concentrations[10]. As a resultant, prematurity is associated with decreased antioxidant reserve. Previously, it has been shown that markers of oxidative stress were reduced in girls compared to boys during the first 24 hours of postnatal life[11]. Furthermore, enzymes involved in glutathione (GSH) oxidation and recycling were increased in girls compared to boys, possibly reflecting increased oxidant removal and more efficient recycling of GSH by girls[12].

GSH is the main non-enzymatic intracellular antioxidant. GSH is a tripeptide synthesized from the amino acids glutamate, cysteine and glycine. It is synthesized in virtually all tissues, but is mainly produced in the liver and erythrocytes with erythrocytic concentrations being in the millimolar range[13]. Erythrocytes likely play a major role in antioxidant defense by being a physiological source of GSH and by taking up ROS[14 15]. GSH exerts its antioxidant properties by 1) oxidation of GSH to its dimeric form (GSSG) thereby reducing hydrogen peroxide by transferring the energy of the reactive peroxides to the sulphydryl group from the cysteine residue, 2) functioning as an essential cofactor for enzymes such as GSH-S-transferases (GST) which catalyses binding with lipid oxidation products, and 3) by direct binding of toxic metals to its sulphydryl group. While preterm infants show diminished availability of other components of the antioxidant defense systems, the GSH concentrations in cord blood of preterm infants resemble those found in term infants[14 16 17]. GSH concentrations, however, fall rapidly after birth in preterm infants[18]. Whether this drop in concentration, at the time when antioxidants are needed most, is due to immaturity of the antioxidant system is not studied yet.

To determine whether gender, GA and BW are correlated with GSH synthesis and recycling, we performed a retrospective analysis of all available data of previous studies performed by our research group. We hypothesized that longer gestation, higher birth weight and female gender are associated with higher GSH synthesis and more efficient GSH recycling.

SUBJECTS AND METHODS

This analysis is a secondary analysis of studies previously performed at the Neonatal Intensive Care Unit of the Erasmus MC - Sophia Children's Hospital (Rotterdam, The Netherlands). All studies were investigator-initiated, without external funding. The study protocols were approved by the Erasmus MC Medical Ethical Committee. Written parental informed consent was obtained for all infants.

Glutathione synthesis rates

Subjects

Very low birth weight (VLBW; birth weight <1500g) infants from five studies on GSH kinetics performed between October 2005 and October 2011 were included in the analysis[18-21, **Chapter 8**]. VLBW infants with a central venous and arterial catheter for clinical purposes were eligible for the studies. Exclusion criteria were major congenital anomalies, chromosome defects, metabolic diseases, and endocrine, renal or hepatic disorders. Data on administration of antenatal steroids, gestational age (based on early fetal ultrasonography or on the date of last menstrual period), birth weight, gender, mode of delivery, Apgar scores, umbilical cord pH, Clinical Risk Index for Babies (CRIB) score[22] and other demographic characteristics were recorded.

Blood sampling and analytical methodology

On the second postnatal day, a primed (20 to 40 µmol/kg) continuous (20 µmol/(kg•h)) infusion of [1-^{13}C]glycine or [U-^{13}C]glycine (99% enriched; Cambridge Isotope Laboratories, Andover, MA, USA; GMP, sterility and pyrogenicity tested; dissolved in 0.9% saline by the hospital pharmacist) was administered during 6 to 8 hours using a Perfusor fm infusion pump (B|Braun Medical B.V., Oss, the Netherlands). Blood was sampled from an indwelling arterial catheter and collected in EDTA containing microtainers. The blood samples were immediately put on melting ice and centrifuged at 3500 x g for 10 min. The plasma fraction was removed, and the lower layer (containing primarily erythrocytes) was reconstituted to its original volume with ice-cold distilled water and stored at -80°C until further analysis. To calculate the fractional synthesis rates (FSR) and absolute synthesis rates (ASR), the concentrations and enrichments of GSH and its precursor glycine were determined in the erythrocytes according to previously described mass spectrometric analysis[23 24].

GSH-GSSG ratio

Subjects

Preterm infants with a GA < 32 weeks (based on early fetal ultrasonography or on the date of last menstrual period) who participated in a double-blind study on optimal oxygen concentration during resuscitation and from whom GSH-GSSG ratio was determined on the first postnatal day

were included in this analysis[25]. Exclusion criteria and data collection were similar as described for GSH synthesis rates.

Blood sampling

Within 24 hours of birth, blood samples were drawn from an arterial catheter or via a heel prick. Blood samples were collected in heparinized microtainers and immediately placed on melting ice. After centrifugation at 3500 x g for 10 min, the plasma fraction was removed and the lower layer, containing primarily erythrocytes, was reconstituted to regain the original volume with ice-cold destilled water and stored at -80°C until analysis.

Analytical methodology

An aliquot of 50 µL erythrocytes suspension was diluted with 50 µL of water and 10 µL of internal standard solution was added. The internal standard consisted of 1 mM cycloleucine (Sigma, St Louis, USA) and 1.5 mM 13C2,15N-GSH (Cambridge Isotope Laboratories Buchem, Apeldoorn, The Netherlands) dissolved in 0.1 M HCl (37% v/v; Merck, Darmstadt, Germany). The erythrocytes were disrupted by freezing the mixture in liquid nitrogen and thawing at room temperature. After three freeze/thaw cycles, the samples were sonicated for 5 minutes. Then, 50 µL of 2M perchloric acid (70% v/v; Merck, Darmstadt, Germany) was added to the samples, and they were placed on ice. After 10 minutes, the samples were centrifuged for 10 min at 3000 rpm. Next 50 µL 200 mM N-ethylmaleimide (NEM; Sigma, St Louis, USA) was added to the supernatant and the mixture was transferred to a costar spin filter (0.22 µm). After centrifugation for 5 min at 1500 rpm, the filtrate was incubated for 4 hours at 40°C.

Stock solutions of GSH and GSSG (Sigma, St Louis, USA) in 0.1 M HCl were prepared at concentrations of 8 mM and 1 mM, respectively and stored at -80°C. A calibration curve was prepared by diluting the stock solution with 0.1M HCl to achieve a concentration range of 0.1-2 mM and 0.1-100 µM for GSH and GSSG respectively. 100 µL of each diluted solution was mixed with 20 µL of internal standard and 50 µL of 200 mM NEM in water and incubated for 4 hours at 40°C before analysis.

Analysis was performed on a Velos Pro LC-linear ion trap system consisting of an autosampler (Accela AS autosampler), an HPLC pump (Accela 600 Pump), fitted with a Hypersil gold-column (C18, 50 x 2.1 mm, 1.9µm) (Thermo Fischer, Rockford, USA). The samples were kept at 10°C upon analysis. Aliquots of 5 µL of each sample were injected in triplicate. The column oven temperature was set to 26°C. The LC-flow rate was 200 µL/min, and a multistep gradient with 0.05% heptafluorobutyric acid (Thermo Fisher, Rockford, USA) and 0.05% trifluoroacetic acid (Applied Biosystems Limited, Warrington, UK) as solvent A and Acetonitrile (Biosolve, Valkenswaard, The Netherlands) as solvent B was used to separate the components of interest. After 15 injections, the column was rinsed with solvent B to prevent peak shifting, followed by two blank injections with the analysis gradient before continuing the analysis of samples. A HESI interface was used to introduce the samples to the Velos Pro ion trap MS. The capillary temperature was set to

300°C, and the source heater to 350°C. The sheath gas flow and auxiliary gas flow were 84 and 34, respectively. The components of interest, GSH, GSSG, GSH-NEM and cycloleucine were measured in zoom mode. During all segments, mass range was set to normal; scan rate to zoom; full scan; positive polarity and the data were recorded in profile mode.

The results obtained by mass spectrometry were used for calculation of the GSH/GSSG ratio. [13C2,15N]-GSH served as an internal standard for GSH, and cycloleucine as an internal standard for GSSG. When a pool consists of both unlabelled GSH (A) and [13C2,15N]-GSH (B), three isotopologues of GSSG are formed. The distribution between unlabelled GSSG (AA), [13C2,15N]-GSSG (AB) and [13C4,15N2]-GSSG (BB) can be predicted from the ratio of GSH to [13C2,15N]-GSH in the pool using a statistical equation. If A<B, AA = (A/B) x 0.25 x AB. However, if A>B, AA = (A/2B-0.25) x AB. Thus, by measuring the ratio unlabeled GSH to [13C2,15N]-GSH and the concentration of [13C2,15N]-GSSG, the amount of unlabeled GSSG that was formed in vitro (i.e. after addition of [13C2,15N]-GSH) can be calculated. After subtraction of this amount from the concentration unlabeled GSSG measured in the sample, the true value in vivo is obtained. The GSH concentration was corrected using a calibration graph and an isotopologue as internal standard. Dimerization and incomplete derivatization occur when using NEM as a reacting reagent. In both processes, losses and inefficiencies are accounted for by the same percentage loss of [13C2,15N]-GSH, so the ratio GSH: [13C2,15N]-GSH remains constant. In conclusion, [13C2,15N]-GSH is not only added to be able to calculate the GSSG formation in vitro, but is also necessary for correct quantification of GSH.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20 (IBM Corporation, Armonk, NY, USA). The Mann-Whitney test was used to determine differences in GSH kinetics for nominal determinants, like gender. For continuous determinants, like GA, correlations with GSH kinetics were determined with Spearman's correlation.

RESULTS

In total, 109 infants were included in the analysis of GSH concentration and synthesis rate and 75 different infants were included in the analysis of the GSH-GSSH ratio. **Table 1** depicts the general demographic data of both groups.

Table 1: Clinical and obstetric characteristics.

	GSH synthesis rates	GSH-GSSG ratio
N (male : female)	109 (73 : 36)	75 (34 : 41)
Gestational age (weeks)	27 1/7 ± 1 6/7	27 3/7 ± 2
Birth weight (g)	914 ± 214	980 ± 322
Birth weight Z-score	-1.0 ± 1.3	-1.0 ± 1.4
Umbilical cord pH	7.25 ± 0.13	7.29 ± 0.10
Apgar score at 5 min	8 ± 2	8 ± 1
CRIB score#	4.5 ± 3.2	4.0 ± 3.5

Data are depicted as mean ± SD. #The CRIB score (Clinical Risk Index for Babies) indicates the degree of illness[22]. The score has a maximum of 23 points and is positively correlated with the severity of illness.

Glutathione concentration and synthesis rates on postnatal day 2

GSH concentration and synthesis rates were not different between males and females (**Figure 1**). Both gestational age and birth weight were also not correlated with GSH concentration and synthesis rates (**Figure 2**). We also explored the effect of other potential modulators. Administration of antenatal steroids, mode of delivery or CRIB score were not correlated with differences in GSH kinetics (data not shown).

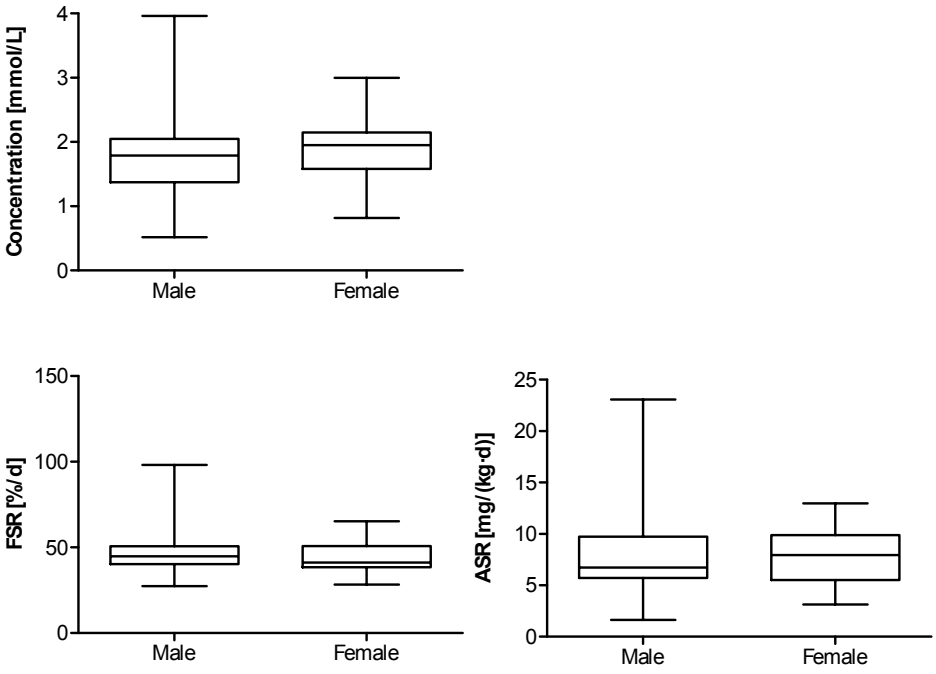

Figure 1: Gender and GSH synthesis.
Data are shown as box-plots. Glutathione concentration, fractional synthesis rate (FSR) and absolute synthesis rate (ASR) were not different between males and females.

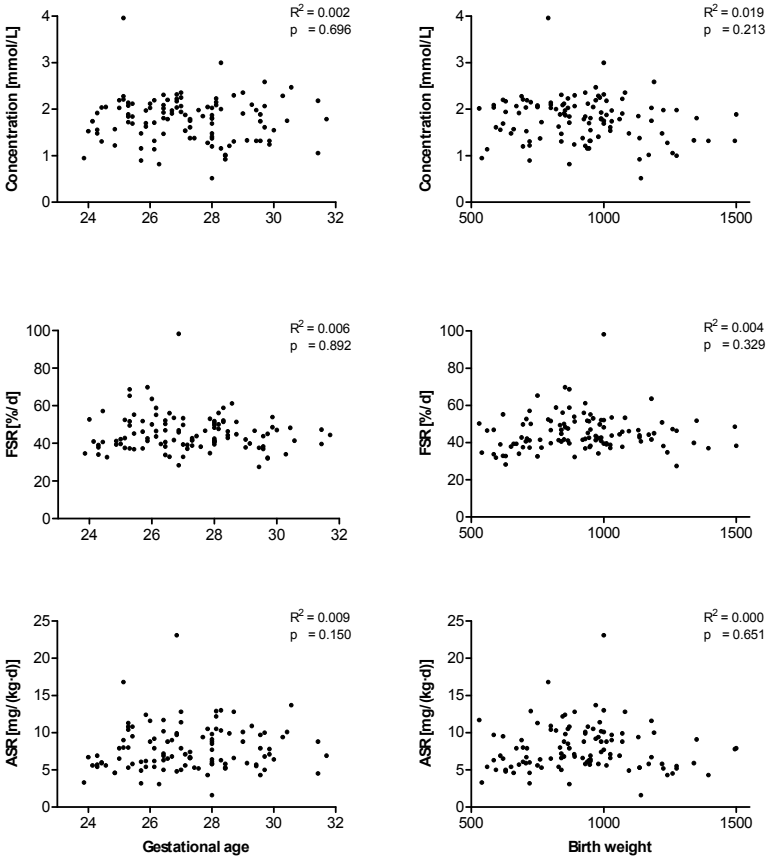

Figure 2: Correlation of gestational age and birth weight with GSH synthesis.
Correlations were analyzed using Spearman's correlation. FSR = fractional synthesis rate, ASR = absolute synthesis rate.

GSH-GSSG ratio on the first postnatal day

GSH-GSSG ratio was slightly lower in females than in males (**Figure 3**). The difference in the ratio was small (females 274 ± 53 and males 311 ± 74, p 0.024). Both gestational age and birth weight (**Figure 4**) were not correlated with GSH-GSSG ratio.

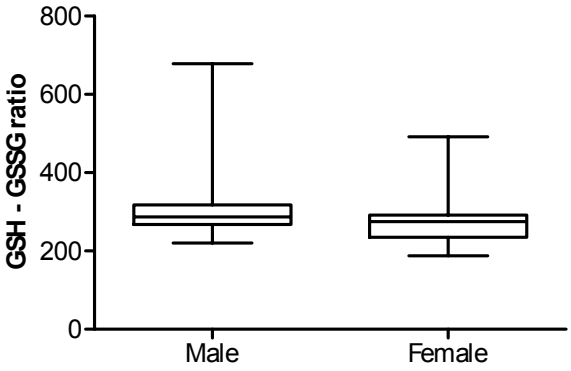

Figure 3: Gender and GSH-GSSG ratio.
Data are shown as box-plots. GSH – GSSG ratio was significantly lower in females compared to males ($p = 0.024$).

The time interval between administration of antenatal steroids and birth, mode of delivery, umbilical cord pH and Apgar scores were not correlated with differences in GSH kinetics (data not shown). CRIB score, however, was correlated with GSH-GSSG ratio ($R^2 = 0.062$, $p = 0.035$). The low R^2 indicates that the contribution of the CRIB score to the GSH-GSSG ratio is, although statistically significant, not very relevant.

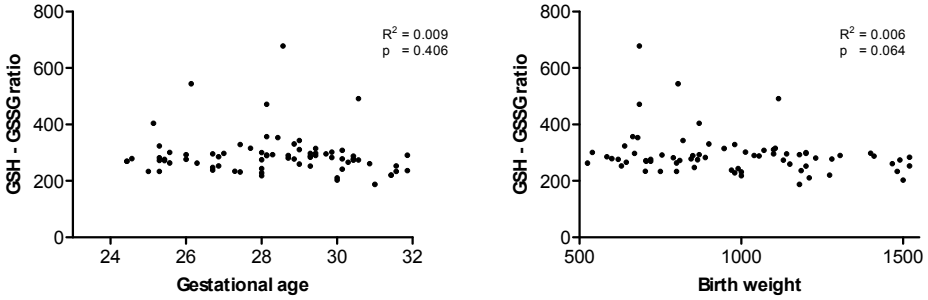

Figure 4: Correlation of gestational age and birth weight with GSH-GSSG ratio.
Correlations were analyzed using Spearman's correlation.

DISCUSSION

Gender, gestational age, birth weight and neonatal morbidities related to oxidative stress are important prognostic factors for long-term outcome of preterm infants. We hypothesized that gender, gestational age and birth weight would influence GSH synthesis and GSH oxidation. In this study, we demonstrate that GSH concentration and synthesis rates are unaffected by these factors, but GSH-GSSG ratio was slightly but significantly lower in females than in males.

GSH is the most important non-enzymatic intracellular antioxidant. At birth, GSH concentrations of preterm infants resemble those found in term infants, but decrease rapidly during the first days of life and become significantly lower than in term neonates[14 16-18 26 27]. This rapid decline is probably caused by increased consumption of GSH due to oxidative stress, but it has also been suggested that GSH synthesis is insufficient in preterm infants due to immaturity of the enzymatic apparatus. However, we did not find a correlation between gestational age and GSH synthesis. GSH synthesis is also dependent on adequate substrate for GSH synthesis, but increasing amino acid intake or supplying preterm infants with increased energy for protein synthesis do not seem to affect GSH synthesis (discussed in **Chapters 7 and 8**). Mean FSR of the 109 included preterm infants was 45%/d, with a ranges of 27 - 98%/d, demonstrating large interindividual differences. The mechanism resulting in these large differences remains unclear.

Interestingly, the GSH-GSSG ratio was slightly lower in females than in males, which is considered to be indicative of higher exposure to oxidative stress as more GSH is oxidized to GSSH. Our finding is in contrast to previously reported increased glutathione peroxidase (GPx) and glutathione reductase (GR) and reduced markers of oxidative stress in girls compared to boys[11 12]. Those findings suggest a more efficient recycling of GSH resulting in decreased oxidative stress in girls, which might be a possible mechanism explaining the better outcome of girls. Since we are not aware of any factors that would impose females to more oxidative stress than males in our study and the difference was not very large, the difference between males and females may very well be a variation from the mean. Unfortunately, we were not able to relate the GSH-GSSH ratio to other markers of oxidative stress or measure enzyme activities of GPx and GR to unravel this issue. Availability of blood samples for research is limited in the studied population.

GA and BW were not correlated with differences in GSH-GSSG ratio. Preterm birth is often associated with fetal or maternal morbidities, such as preeclampsia or chorioamnionitis, which are linked to increased oxidative stress[28 29]. Possibly, oxidative stress is already so highly increased in preterm infants, that immaturity, as reflected by GA or BW, does nor further add to this burden. We did demonstrate a small association between the CRIB score, reflecting illness within the first 12 hours after birth, and the GSH-GSSG ratio.

Previously, it has been shown that administration of antenatal steroids resulted in increased superoxide dismutase (SOD) and catalase activity (CAT), both important enzymatic antioxidants[11]. Also, enzymes involved in the GSH redox cycle were increased upon antenatal steroid administration, resulting in decreased GSH-GSSG ratio. In our study, administration and the timing of administration of antenatal steroids was not correlated with GSH synthesis. All infants included in the analysis of GSH-GSSG ratio received antenatal steroids, thus the effect of administration versus no administration could not be studied. However, the interval between its administration and birth was not correlated with GSH-GSSG ratio. Also, GSH synthesis was not related to administration of antenatal steroids or the timing of administration.

A possible explanation for the large interindividual differences in GSH synthesis, and also GSH recycling, might be found in genetic differences in the GSH synthesis apparatus. Research in this field is still in its infancy and has been mainly focused on the risk of adult cancer. Polymorphisms in the glutathione-S-transferase (GST) and SOD gene have been associated with the risk of developing infant respiratory distress syndrome, BPD, IVH and ROP in preterm infants[30-32]. Polymorphisms in microsomal epoxide hydrolase (EPHX), involved in the detoxification of epoxides which may be extremely toxic by the induction of oxidative stress, have also been associated with perinatal mortality[33]. Polymorphisms in antioxidant enzymes have indeed been found to alter enzyme activity in response to oxidative stress and might therefore be associated with differences in the ability to counteract oxidative stress[34 35]. It would be interesting to investigate whether polymorphisms in enzymes involved in GSH synthesis might alter GSH synthesis and its response to oxidative stress.

A main limitation of this analysis is that GSH synthesis and GSH-GSSG ratio were measured in different populations and on different postnatal days. Also, we measured GSH-GSSG ratio in erythrocytes and it could be argued whether this is representative for the total GSH-GSSG ratio. Furthermore, we could not related our findings to other markers of oxidative stress and therefore we cannot draw definite conclusions. The mechanisms of oxidative stress and antioxidant defense in relation to maturation and morbidity remain of high interest in perinatal medicine. Future studies should measure GSH synthesis, recycling, enzyme activity and levels of oxidative stress simultaneously and in different tissues or compartments in order to uncover determinants of GSH kinetics in preterm infants.

In conclusion, differences in outcome related to gestational age, birth weight and gender cannot be explained by differences in GSH synthesis rates. The GSH-GSSG ratio is lower in girls compared to boys, but the implications of this finding need to be determined. Further research into modulators of GSH kinetics should focus on, amongst others, (epi)genetic differences in components of the antioxidant apparatus and measurement of multiple parameters of oxidative stress simultaneously.

REFERENCES

1. Georgeson GD, Szony BJ, Streitman K, Varga IS, Kovacs A, Kovacs L, et al. Antioxidant enzyme activities are decreased in preterm infants and in neonates born via caesarean section. *Eur J Obstet Gynecol Reprod Biol* 2002;103(2):136-9.
2. Buhimschi IA, Buhimschi CS, Pupkin M, Weiner CP. Beneficial impact of term labor: nonenzymatic antioxidant reserve in the human fetus. *Am J Obstet Gynecol* 2003;189(1):181-8.
3. Comporti M, Signorini C, Leoncini S, Buonocore G, Rossi V, Ciccoli L. Plasma F2-isoprostanes are elevated in newborns and inversely correlated to gestational age. *Free radical biology & medicine* 2004;37(5):724-32.
4. Buonocore G, Perrone S, Longini M, Vezzosi P, Marzocchi B, Paffetti P, et al. Oxidative stress in preterm neonates at birth and on the seventh day of life. *Pediatr Res* 2002;52(1):46-9.
5. Saugstad OD. The oxygen radical disease in neonatology. *Indian J Pediatr* 1989;56(5):585-93.
6. Incidence of and Risk Factors for Neonatal Morbidity after Active Perinatal Care: Extremely Preterm Infants Study in Sweden (EXPRESS). *Acta Paediatr* 2010;99(7):978-92.
7. Stoll BJ, Hansen NI, Bell EF, Shankaran S, Laptook AR, Walsh MC, et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics* 2010;126(3):443-56.
8. Ambalavanan N, Carlo WA, Tyson JE, Langer JC, Walsh MC, Parikh NA, et al. Outcome trajectories in extremely preterm infants. *Pediatrics* 2012;130(1):e115-25.
9. Tyson JE, Parikh NA, Langer J, Green C, Higgins RD. Intensive care for extreme prematurity--moving beyond gestational age. *The New England journal of medicine* 2008;358(16):1672-81.
10. Davis JM, Auten RL. Maturation of the antioxidant system and the effects on preterm birth. *Semin Fetal Neonatal Med* 2010.
11. Vento M, Aguar M, Escobar JJ, Arduini A, Escrig R, Brugada M, et al. Antenatal Steroids and Antioxidant Enzyme Activity in Preterm Infants: Influence of Gender and Timing. *Antioxid Redox Signal* 2009.
12. Hamon I, Valdes V, Franck P, Buchweiller MC, Fresson J, Hascoet JM. [Gender-dependent differences in glutathione (GSH) metabolism in very preterm infants]. *Arch Pediatr* 2011;18(3):247-52.
13. Darmaun D, Smith SD, Sweeten S, Sager BK, Welch S, Mauras N. Evidence for accelerated rates of glutathione utilization and glutathione depletion in adolescents with poorly controlled type 1 diabetes. *Diabetes* 2005;54(1):190-6.
14. Ahola T, Levonen AL, Fellman V, Lapatto R. Thiol metabolism in preterm infants during the first week of life. *Scand. J. Clin. Lab. Invest.* 2004;64(7):649-58.
15. Giustarini D, Milzani A, Dalle-Donne I, Rossi R. Red blood cells as a physiological source of glutathione for extracellular fluids. *Blood Cells Mol Dis* 2007;40(2):174-9.
16. Jain A, Mehta T, Auld PA, Rodrigues J, Ward RF, Schwartz MK, et al. Glutathione metabolism in newborns: evidence for glutathione deficiency in plasma, bronchoalveolar lavage fluid, and lymphocytes in prematures. *Pediatr. Pulmonol.* 1995;20(3):160-6.
17. Jean-Baptiste D, Rudolph N. Sequential postnatal changes in erythrocyte glutathione and sulfhydryl content: a possible adaptational response to the extrauterine environment. *Biol. Neonate* 2003;84(2): 142-6.
18. Rook D, Te Braake FW, Schierbeek H, Longini M, Buonocore G, van Goudoever JB. Glutathione Synthesis Rates in Early Postnatal Life. *Pediatr Res* 2010;67(4):407-11.
19. Te Braake FW, Schierbeek H, de Groof K, Vermes A, Longini M, Buonocore G, et al. Glutathione synthesis rates after amino acid administration directly after birth in preterm infants. *Am J Clin Nutr* 2008; 88(2):333-9.
20. Te Braake FW, Schierbeek H, Vermes A, Huijmans JG, van Goudoever JB. High-dose cysteine administration does not increase synthesis of the antioxidant glutathione preterm infants. *Pediatrics* 2009; 124(5):e978-84.

21. Te Braake FW, Schierbeek H, de Groof K, Huijmans JGM, Longini M, Buonocore G, et al. Glutathione synthesis rates and oxidative stress in extremely low birth weight infants in the first week of life. *Unpublished*.
22. The CRIB (clinical risk index for babies) score: a tool for assessing initial neonatal risk and comparing performance of neonatal intensive care units. The International Neonatal Network. *Lancet* 1993; 342(8865):193-8.
23. Schierbeek H, Te Braake F, Godin JP, Fay LB, van Goudoever JB. Novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2007;21(17):2805-12.
24. Schierbeek H, Rook D, te Braake FW, Dorst KY, Voortman G, Godin JP, et al. Simultaneous analysis of (13)C-glutathione as its dimeric form GSSG and its precursor [1-(13)C]glycine using liquid chromatography/isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* 2009;23(18):2897-902.
25. Rook D, Schierbeek H, van der Eijk A, Longini M, Buonocore G, Vento M, et al. Resuscitation of very preterm infants with 30% vs. 65% oxygen at birth: study protocol for a randomized controlled trial. *Trials* 2012;13(1):65.
26. Clahsen PC, Moison RM, Holtzer CA, Berger HM. Recycling of glutathione during oxidative stress in erythrocytes of the newborn. *Pediatr Res* 1992;32(4):399-402.
27. Frosali S, Di Simplicio P, Perrone S, Di Giuseppe D, Longini M, Tanganelli D, et al. Glutathione recycling and antioxidant enzyme activities in erythrocytes of term and preterm newborns at birth. *Biol Neonate* 2004;85(3):188-94.
28. Cheah FC, Pillow JJ, Kramer BW, Polglase GR, Nitsos I, Newnham JP, et al. Airway inflammatory cell responses to intra-amniotic lipopolysaccharide in a sheep model of chorioamnionitis. *Am J Physiol Lung Cell Mol Physiol* 2009;296(3):L384-93.
29. Raijmakers MT, Roes EM, Poston L, Steegers EA, Peters WH. The transient increase of oxidative stress during normal pregnancy is higher and persists after delivery in women with pre-eclampsia. *Eur J Obstet Gynecol Reprod Biol* 2008;138(1):39-44.
30. Poggi C, Giusti B, Vestri A, Pasquini E, Abbate R, Dani C. Genetic polymorphisms of antioxidant enzymes in preterm infants. *J Matern Fetal Neonatal Med* 2012;25 Suppl 4:131-4.
31. Manar MH, Brown MR, Gauthier TW, Brown LA. Association of glutathione-S-transferase-P1 (GST-P1) polymorphisms with bronchopulmonary dysplasia. *J Perinatol* 2004;24(1):30-5.
32. Oretti C, Marino S, Mosca F, Colnaghi MR, De Iudicibus S, Drigo I, et al. Glutathione-S-transferase-P1 I105V polymorphism and response to antenatal betamethasone in the prevention of respiratory distress syndrome. *European journal of clinical pharmacology* 2009;65(5):483-91.
33. Raijmakers MT, de Galan-Roosen TE, Schilders GW, Merkus JM, Steegers EA, Peters WH. The Tyr113His polymorphism in exon 3 of the microsomal epoxide hydrolase gene is a risk factor for perinatal mortality. *Acta obstetricia et gynecologica Scandinavica* 2004;83(11):1056-60.
34. Komina AV, Korostileva KA, Gyrylova SN, Belonogov RN, Ruksha TG. Interaction between single nucleotide polymorphism in catalase gene and catalase activity under the conditions of oxidative stress. *Physiological research / Academia Scientiarum Bohemoslovaca* 2013;61(6):655-8.
35. Bresciani G, Gonzalez-Gallego J, da Cruz IB, de Paz JA, Cuevas MJ. The Ala16Val MnSOD gene polymorphism modulates oxidative response to exercise. *Clinical biochemistry* 2012.

Chapter 12

Defining hazards of supplemental oxygen therapy in neonatology using the FMEA tool

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ABSTRACT

Introduction

Both hyperoxia and hypoxia can have detrimental consequences for preterm infants. The manual control of the oxygenation is difficult and oxygen saturation levels measured by a pulse oximeter (SpO_2) are frequently outside of the target range. The objective of this study was to evaluate hazards in the process of supplemental oxygen therapy in very preterm infants hospitalized in a Dutch Neonatal Intensive Care Unit prospectively.

Methods

A Failure Mode and Effects Analysis (FMEA) was conducted by a multidisciplinary team. This team identified, evaluated, and prioritized hazards of supplemental oxygen therapy in preterm infants. After accrediting 'hazard scores' for each step in this process, recommendations were formulated for the main hazards.

Results

Performing the FMEA took seven meetings of two hours. The top ten hazards could all be categorized to three main topics: incorrect adjustment of the fraction of inspired oxygen (FiO_2), incorrect alarm limits for SpO_2, and incorrect pulse-oximetry alarm limits on patient monitors for temporarily use. The FMEA culminated in recommendations in both educational and technical directions. These included suggestions for (changes in) protocols on alarm limits and manual FiO_2 adjustments, education of NICU staff on hazards of supplemental oxygen, and technical improvements in respiratory devices and patient monitors.

Conclusions

The FMEA prioritized flaws in the process of supplemental oxygen therapy in very preterm infants. Thanks to the structured approach of the analysis by a multidisciplinary team, several recommendations were made. These recommendations are currently implemented in the study's centre.

INTRODUCTION

As a result of on-going developments in neonatal care, long-term outcomes of preterm infants are still improving[1]. However, innovations in neonatal intensive care units (NICUs) are accompanied with an increased complexity of daily practice. In complex environments like NICUs, where people from different disciplines work in shifts and with high-tech equipment, the occurrence of errors is, unfortunately, undeniable.

In the year 2000, the Institute of Medicine published 'To Err Is Human: Building a Safer Health System'[2]. Thanks to this report, patient safety and the reduction of medical errors obtained major priority in health care. In the NICU, where care focuses on a specific and very vulnerable patient group, errors may have even more impact than in other disciplines of health care. Therefore, although much work has already been performed, each opportunity to increase (patient) safety in the NICU should be seized[3].

In 2005, a 'Neonatology System for Analysis and Feedback on medical Events (NEOSAFE)' was introduced in the Netherlands to establish specialty-based learning from (near-) misses[4]. The (near-) misses are reported by NICU personnel on a voluntary basis. In the NICU of the Erasmus Medical Center-Sophia Children's Hospital in Rotterdam, 909 (near-) misses were reported in 2010. While only 58 (6%) of the reports were related to 'supplemental oxygen therapy', these 58 reports were responsible for 90% of the (near-) misses that were categorised as 'most risky'.

In supplemental oxygen therapy, a gas mixture with >21% of oxygen is supplied to the patient via (mechanical) ventilation. Due to the immaturity of the preterm infants' lungs, supplemental oxygen therapy is often needed immediately after birth to reach and maintain adequate oxygenation. Unfortunately, supplemental oxygen therapy has a very narrow therapeutic range. Both too high and too low blood oxygen levels may have severe consequences for the outcome of these infants[5].

Literature shows that manual control of the oxygenation is difficult and that oxygen saturation levels measured by a pulse oximeter (SpO_2) are frequently outside of the target range[6-9]. Therefore, the aim of this study was to evaluate hazards in the process of supplemental oxygen therapy in very preterm infants hospitalized on the NICU prospectively, and to provide recommendations for improvement.

To evaluate the process, a 'Failure Mode and Effects Analysis (FMEA)' tool was used. To prevent failure modes in high risk industries, like aviation, and nuclear power plants, engineers analyse products and processes for potential hazards. Since the mid-1960s, formal 'Failure Mode and Effects Analyses (FMEAs)' have been performed to standardize the approach in failure mode detection. Although performing an FMEA is often considered as time-consuming and needs organizational commitment, the method is a useful tool for structural analysis, identification of (unnoticed) errors, and has been demonstrated to increase safety[10]. Unfortunately, FMEAs could not be applied easily to healthcare situations. Therefore, several adaptations on the methodology of FMEAs have been considered, developed and used[11-13].

METHODS

On the NICU of the Erasmus Medical Center-Sophia Children's Hospital in Rotterdam, the Netherlands, an FMEA on supplemental oxygen therapy in very preterm infants was performed. Approval from the hospital research ethics board was not necessary because no patients were involved in the study.

The Erasmus MC has a level-III-c[14] NICU with 30 beds divided over three subunits. The nursing staff works in shifts of 8 hours. After each shift, half an hour of 'handover' takes place. During the handover, nurses inform each other verbally about the (clinical) situation of the infants. Moreover, relevant patient data are collected and validated at least once per hour in the Patient Data Management System (PDMS) by the nursing and/or medical staff.

The FMEA consisted of five steps (**Table 1**)[13 15]. With the FMEA team, several plenary sessions were held to complete each of the steps. Besides the actual FMEA-team, other employees were asked to join in a separate panel of FMEA advisors: the FMEA support-team (as suggested by Ashley et al.[16]). This support-team was included in e-mail conversations, but was not present at the plenary meetings.

Table 1: The 5 steps forming the FMEA.

Step	Object
1	Define a topic
2	Assemble a multidisciplinary team
3	Analyse the process by dividing the process in (sub)steps
4	Perform Hazard analysis
	§ Identify 'Hazards', i.e. potential 'failure modes', and possible 'causes' and 'effects' of these failure modes
	§ Accredit risk priority numbers (RPN) for each of the identified Hazards
	§ Determine the top ten hazards
5	Develop recommendations to resolve Hazards

The FMEA-team analysed the process of supplemental oxygen therapy and subdivided it in (sub)sub-steps. For each (sub)sub-step potential 'failure modes', and possible 'causes' and 'effects' of these failure modes were identified. The team was encouraged to identify as many failure modes, causes and effects as possible by brainstorming.

After defining the 'hazards', i.e. the 'failure mode-cause-effects combinations', each team member individually accredited three scores for each of these hazards by using the Hazard Scoring Matrix (**Table 2**). The scores were provided for 'severity (S)', 'likelihood of occurrence (O)', and 'detectability (D)'. Based on these scorings, for each hazard a risk priority number (RPN = S x O x D) was calculated. For each hazard the 'overall RPN' was calculated by taking the mean of all RPN's that were given to this specific hazard by each of the individual team members. The

Table 2: Hazard Scoring Matrix.

Rating	Severity of hazard (S)	Occurrence of hazard (O)	Detectability of hazard (D)
1-3	No consequences	Rare	Always detected
4-6	Minor consequences	Occasional	Probably detected
7-8	Temporary consequences	Frequent	Low probability of detection
9	Permanent consequences	Often	Probably undetected
10	Fatal consequences	Always	Undetected

The Risk Priority Number (RPN) is calculated by multiplying the Severity (S), Occurrence (O) and detectability (D) of the hazard. For example, a hazard that has "minor consequences" (rate 4), that occurs "often" (rate 9) and that has a "low probability of detection" (rate 7) has a RPN of 4 x 9 x 7 = 252.

ten hazards with the highest overall RPN were defined as the "top ten hazards". Finally, the team made recommendations to minimize the hazards.

RESULTS

Step 1. Defining the topic

First, the topic for the FMEA was defined in detail. It was decided to focus on supplemental oxygen therapy for very preterm infants with a postmenstrual age (PMA) <30 weeks, who are admitted to the NICU of the Erasmus MC. In the NICU, every bed space has a separate outlet in the wall for oxygen. Devices for respiratory support are connected to these outlets with a tube. It was decided to include the devices for respiratory support in the FMEA, but to exclude the supply of oxygen from the central oxygen storage to the device for respiratory support.

Step 2. Team assembly

A multidisciplinary team of six members and two team leaders was composed. The members of the team (1 neonatologist, 1 nurse practitioner, 2 NICU-nurses, 1 leading NICU-nurse, and 1 PhD student (second author)) were instructed about the FMEA procedure by the team leaders (first author), and by a consultant for quality improvement and patient safety from the directorate patient care of the study's center. The team was chosen such that all parties relevant in the process of supplemental oxygen therapy in the NICU were represented. In a four month period, the team had seven meetings of approximately two hours. In between meetings, communication via e-mail was used to spread relevant documents. The FMEA support team was included in the e-mail conversation, and existed of seven members (five employees of the NICU, and the third and fourth author).

Step 3. Process analysis

During the process analysis, all process-steps involved in supplemental oxygen therapy were defined. Four main steps were determined:

- Process-step 1: Nurse prepares admission of neonate to NICU
- Process-step 2: Neonate arrives at NICU
- Process-step 3: Neonate is hospitalized on NICU
- Process-step 4: Neonate is discharged from NICU

To prevent unnecessary complexity of the FMEA, only situations that were directly related to supplemental oxygen therapy in preterm infants with a GA <30 weeks admitted to the NICU were included in the analysis. Where applicable, the process-steps were divided in (sub)sub-steps. For example, for process-step 3 "Neonate is hospitalized on NICU", eleven sub-steps were defined (**Figure 1**).

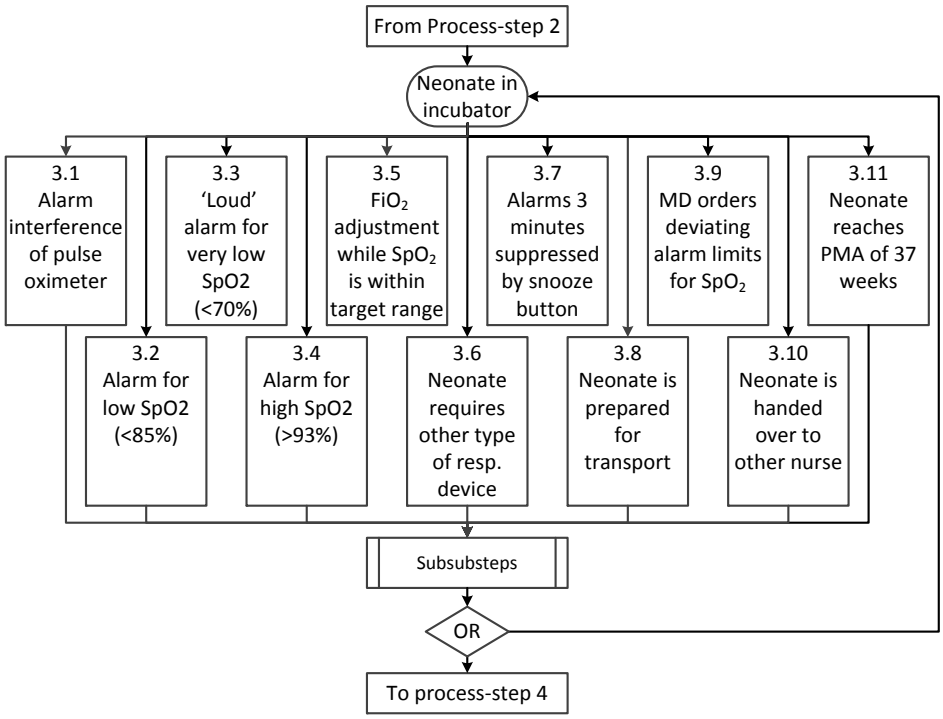

Figure 1: Substeps of process step 3: 'Neonate is hospitalized on NICU'. Subsubsteps are not shown in detail.

Step 4. Hazard analysis

In step four of the FMEA, the actual hazard analysis was performed. In total, 134 hazards were defined. The overall RPN ranged from a minimum score of 45 to the highest RPN of 507. The 10 hazards with the highest overall RPN are shown in **Table 3**. These hazards were all related to

Table 3: Top 10 hazards.

#	Hazard	Category	Overall RPN	Action	Recommendation
1	Too slow reduction of FiO_2, resulting in hyperoxia	Incorrect FiO_2 adjustments	507	Controlled	• Protocol on manual FiO_2 adjustments
2	No adjustment of SpO_2 alarm limits when reaching GA of 37 weeks, resulting in hypoxia	Incorrect alarm limit settings	502	Eliminated	• Technical improvements in patient monitors
3	No reduction of FiO_2, resulting in hyperoxia	Incorrect FiO_2 adjustments	501	Controlled	• Protocol on manual FiO_2 adjustments • Education on hazard of supplemental oxygen therapy
4	Incorrect alarm limits of monitor during transport, resulting in hyperoxia or hypoxia	Incorrect alarm limit settings in temporarily monitor	488	Eliminated	• 'Check alarm limits' is added to the checklist used before transport
5	No adjustment of SpO_2 alarm limits when going from room air to supplemental oxygen, resulting in hyperoxia	Incorrect alarm limit settings	486	Eliminated	• Technical improvements in respiratory devices and patient monitors
6	No adjustment of SpO_2 alarm limits when reaching GA of 37 weeks, resulting in unnecessary alarms for hyperoxia	Incorrect alarm limit settings	434	Eliminated	• Technical improvements in patient monitors
7	Too fast increase FiO_2, resulting in hyperoxia	Incorrect FiO_2 adjustments	408	Controlled	• Protocol on manual FiO_2 adjustments • Education on potential hazard of supplemental oxygen therapy
8	Physician does not record deviating alarm limits	Incorrect alarm limit settings	402	Temporarily accepted	• Create awareness for the need to fill in patient records completely and correctly
9	Too fast reduction of FiO_2, resulting in hypoxia	Incorrect FiO_2 adjustments	402	Controlled	• Protocol on manual FiO_2 adjustments • Education on potential hazard of supplemental oxygen therapy
10	Nurse does not record adjustments of alarm limits in PDMS	Incorrect alarm limit settings	376	Controlled	• Technical improvements in patient monitors

The Hazard Scoring is represented by the overall Risk Priority Number (RPN), i.e. the mean RPN calculated over all the RPN's given to the hazard by each of the team members.

incorrect adjustment of the fraction of inspired oxygen (FiO_2), incorrect alarm limit settings for SpO_2, or incorrect pulse oximetry alarm limit settings on patient monitors for temporary use.

Incorrect adjustments of FiO_2

Incorrect adjustments of FiO_2 can result in periods of hyperoxia and hypoxia. The severity of this effect is difficult to quantify, but probably depends on the length and frequency of the periods. Incorrect adjustments of FiO_2 are defined by the combination of frequency and step size in FiO_2 adjustments. For example, multiple adjustments with a small step size, or a single adjustment with a large step size may have a similar effect on the patient, thus both situations were defined as 'too quick'.

Too slow decreasing of FiO_2 was thought to result from the fact that the risk for hyperoxia might not be sufficiently realized by the nursing staff and the lack of a protocol describing the step size and timing of FiO_2 adjustments. The lack of a protocol was also thought to be the cause for the 'too quick' adjustments in FiO_2. Another cause for 'too quick' increases was the high workload of the nursing staff. Because the nurse has to perform other tasks (simultaneously), (s)he might adjust FiO_2 without waiting for the result of previous adjustment.

Incorrect pulse oximetry alarm limit settings

Just as incorrect adjustments in FiO_2, no or incorrect adjustment of alarm limits for SpO_2 may also lead to periods of hyperoxia or hypoxia. In the study's centre, the protocol for alarm limits for patients with a GA <37 weeks, prescribes alarm limits of 85-100% when patients are on room air, and 85-93% for FiO_2 >21%. Thus, when the need for oxygen increases from 21% to >21% or vice versa, the alarm limit needs to be adjusted. This protocol for alarm limits is based on, amongst others, a study by Finer & Leone[5]. The causes for incorrect alarm limits were thought to lie in the fact that nurses forget to adjust alarm limits, and that nurses think they do better when they do not make an adjustment in alarm limits because, for instance, (s)he supposes the need for supplemental oxygen will not last long.

Another situation in which incorrect alarm limits can occur is related to conscious deviations from the protocol. In consultation with the physician, nurses can deviate from the protocol for alarm limits for medical reasons. However, when the non-protocolled alarm limits are not recorded, other NICU personnel may not be aware of the deviating alarm limits, and furthermore, cannot detect who decided to adjust the alarm limits for what reason.

When a preterm infant reaches a PMA of 37 weeks, the protocol for alarm limits changes. The protocol for this patient group prescribes 92-100% for patients on room air, and 92-96% for FiO_2 >21%. It often happens that the alarm limits are not adjusted at 37 weeks of PMA, because the responsible nurse is not aware of the actual age of the patient.

Incorrect pulse oximetry alarm limit settings on patient monitors for temporary use

Another hazard was the incorrect settings of alarm limits for SpO_2 on patient monitors for temporary use. Next to the standard monitor belonging to each incubator, there are situations in which the patient is monitored by separate monitors. For instance, during transport, during a surgical procedure, or when an MRI is performed, the patient is disconnected from its 'own' monitor, and connected to another monitor specifically used for transport, surgery, or MRI. These monitors are used for multiple patients. A consequence of the variety in patients is that monitor settings are not always correctly set for the specific patient. The effects of these incorrect settings depend on the actual setting, but can, for instance, cause unnoticed hypoxia or hyperoxia. The reasons for incorrect monitor settings are probably the variety in default settings of the monitors, and the fact that the nurse and/or physician forget to adjust monitor settings when connecting the patient.

Step 5. Develop Risk Reduction Methods

For each hazard in the top ten, it was determined whether the hazard should be 'eliminated', 'controlled' or 'accepted'. Afterwards recommendations were defined. Several of these recommendations were applicable on multiple hazards. For instance, the fact that caregivers do not recognize the potential harm of hyperoxia, could be improved by a structured educational program. In the study's centre, this encompassed the inclusion of supplemental oxygen therapy as a subject in the education of new NICU nurses, and in the obligatory monthly training for all NICU nurses. The implementation of a protocol for FiO_2 adjustments, like described by Chow et al.[17] and Wilkinson & Andersen[18], could be the solution to minimize incorrect FiO_2 adjustment, and increase standardization in patient care.

The oxygen blender used for respiratory support with a nasal prong, or during manual ventilation, is associated with (near-) misses because the pre-set FiO_2 is difficult to read. The possibility of a more user-friendly oxygen blender will be investigated.

The frequency of incorrect alarm limit settings may decrease by introducing technical solutions. In the most extreme solution, the protocol for alarm limits is implemented in the patient monitor to make sure alarm limits are adjusted automatically. However, to be able to realize this, the monitor needs to be informed about the actual FiO_2 supplied to the infant. In the study's centre, the monitors cannot receive information from ventilators yet. Other solutions could be related to automatic reminders in the PDMS to prompt caregivers to verify pre-set alarm limits. Incorrect setting of temporarily used patient monitors will probably be solved by changing the default alarm limits of all monitors to the same, most conservative, settings. Also, checking of the alarm limits is now included in the checklist for transportation of neonates. For each of the recommendations made in the FMEA, individuals responsible for implementing were identified.

DISCUSSION

This article discusses the FMEA performed on supplemental oxygen therapy in very preterm infants admitted to a NICU. The FMEA resulted in a top ten of hazards for which recommendations were suggested. These hazards could be categorized to three topics, namely incorrect adjustment of the fraction of inspired oxygen (FiO_2), incorrect alarm limit settings for oxygen saturation measured by pulse oximetry (SpO_2), and incorrect pulse oximetry alarm limit settings on patient monitors for temporary use.

With respect to FiO_2 adjustments, protocols describing the step size and timing of FiO_2 adjustments are lacking in the study's centre, and FiO_2 adjustments are now highly dependent on the workload, personal training, and opinion of the caregiver. Literature on this topic is scarce and no randomized studies on how and when to adjust the FiO_2 exist. To improve manual FiO_2 adjustment, the FMEA-team will develop a protocol for FiO_2 adjustments based on current literature and expertise. Another recommendation is to educate caregivers on the hazards of both hypoxia and hyperoxia in preterm infants. However, the effect of education may be transient, and therefore, a structural repetitive program will be implemented.

Incorrect alarm limits for SpO_2 may be resolved with technical solutions. First, awareness by using reminders in the PDMS or the patient monitors could be introduced. These reminders will prompt caregivers to verify alarm limits. Ultimately, automated adjustment of alarm limits for SpO_2, based on the actual FiO_2, should be constructed. Unfortunately, currently used respiratory devices and patient monitors are not able to send and receive information to/from each other. Solutions will be discussed with the industry so recommendations can be taken into account for future developments. Finally, the hazard of incorrect settings in patient monitors was tackled by changing the default alarm limits of all monitors to equal, most conservative settings. Also, in the checklist for transportation of neonates, the task 'check the alarm limits' was included.

Implementation of the recommendations has proven to be difficult. Some of the recommendations are not possible to implement immediately due to technical limitations, other recommendations are part of larger changes in the NICU (e.g. implementation of checklists and protocols), and will be implemented in the near future. Certainly, the knowledge and understanding obtained while performing the FMEA will be shared with other NICUs.

The study was performed in one center, and the question remains whether the hazards are generalizable to other NICUs. However, Nghiem et al.[19] showed that 32% of the centers in the US do not have a protocol for SpO_2 alarm limits, which may also implicate absence of protocols for FiO_2 adjustment. Furthermore, in centres that have a protocol for alarm limits, SpO_2 is frequently outside of the alarm limits[6 7 20].

Compared to other processes that were prospectively analysed for hazards, like the administration of medication[21-26], in the process of supplemental oxygen therapy no clear 'endpoint' can be defined. The process of supplemental oxygen therapy is continued until the patient is discharged from the NICU. This complexity made it difficult to quantify the RPN for hazards.

For instance, in the hazard analysis, the severity of potential consequences was scored ranging from 'no consequences to the patient' to 'fatal consequences for the patient'. However, most failure modes in this FMEA could result in either hypoxia or hyperoxia, with unknown (long-term) consequences. It is known that both hypoxia and hyperoxia can cause permanent damage[27], but it is impossible to determine whether this damage will actually occur and whether this damage will be either temporarily, permanent or fatal.

Besides the difficulty of determining the 'occurrence' and 'severity' of hazards, Ashley and Armitage[28] illustrated that determination of RPNs of hazards is also subjective. To minimize subjectivity, it was decided to first determine RPNs individually, and to discuss the results afterwards in a plenary session. Although the RPNs of the hazards differed slightly between individuals, there was overall consensus about the hazards in the final top ten.

To quantify the effects of implementation of recommendations on the (near-) misses, the FMEA-team is dependent of the voluntary reports of these (near-) misses. The number of reports is not only dependent on the actual (near-) misses, but also on the willingness, and focus of caregivers to detect, and report the (near-) misses.[3] Since these reports of (near-) misses thus only provide an indication for the actual number of (near-) misses, it is impossible to compare failure rates before and after this FMEA. However, despite the lack of quantification, performing an FMEA on supplemental oxygen therapy in very preterm infants hospitalized on the NICU provided interesting insights, and is expected to enhance patient safety.

Lessons learned by the FMEA-team

Performing the FMEA was a new experience for most of the team members. Though, despite the inexperienced team, the FMEA was considered as easy to learn, and intuitive method. The discussions in the plenary meetings were very open minded, and, thanks to the multidisciplinary character, diverse ideas and visions were mentioned. Because the FMEA consultant was not related to the neonatology department, she had independent judgement.

The team members all agreed that performing an FMEA is time consuming. One of the largest challenges for the team leaders was to plan appointments for plenary sessions which all of the team members were able to join. The difficulty to organize meetings was partly caused by the varying working shifts of the caregivers. However, despite the time issue, it is expected that the FMEA tool will be used more frequently in the near future for other processes in Neonatal Intensive Care.

Although the FMEA team existed of only eight members, the importance of reporting (near) misses, identifying failure modes, and implementing recommendations in clinical practice, was probably apprehended by a much larger part of the staff. Not only because of the fact that the results of this FMEA were presented to all NICU nurses and physicians, but also because of the monthly education rounds where (near) misses are discussed, amongst others. Hence, the performance of the FMEA for supplemental oxygen therapy may have positive effects on patient safety in general just by creating awareness among the caregivers.

Conclusion

Performing an FMEA on supplemental oxygen therapy in very preterm infants hospitalized on the NICU provided interesting insights. The main hazards were all related to incorrect adjustment of the FiO_2, incorrect alarm limit settings for SpO_2, and incorrect pulse oximetry alarm limit settings on patient monitors for temporary use. Thanks to the structured approach of the FMEA, and the multidisciplinary team, several recommendations for improvement were made and implemented.

REFERENCES

1. Saigal S, Doyle LW. Preterm birth 3: An overview of mortality and sequelae of preterm birth from infancy to adulthood. *The Lancet* 2008;371(9608):261–9.
2. Kohn LT, Corrigan JM, Donaldson MS. *To err is human: building a safer health system*. Washington DC: The National Academy Press, 2000.
3. Edwards W. Patient safety in the neonatal intensive care unit. *Clinics in Perinatology* 2005;32(1):97-106.
4. Snijders C, Van der Schaaf T, Klip H, van Lingen W, Molendijk A. Feasibility and reliability of PRISMA-Medical for specialty-based incident analysis. *Quality and Safety in Health Care* 2009;18(6):486-91.
5. Finer N, Leone T. Oxygen saturation monitoring for the preterm infant: The evidence basis for current practice. *Pediatric research* 2009;65(4):375-80.
6. Hagadorn JI, Furey AM, Nghiem TH, Schmid CH, Phelps DL, Pillers DAM, et al. Achieved versus intended pulse oximeter saturation in infants born less than 28 weeks' gestation: The AVIOx study. *Pediatrics* 2006;118(4):1574-82.
7. Laptook AR, Salhab W, Allen J, Saha S, Walsh M. Pulse oximetry in very low birth weight infants: can oxygen saturation be maintained in the desired range? *Journal of Perinatology* 2006;26:337-41.
8. Sink DW, Hope SAE, Hagadorn JI. Nurse:patient ratio and achievement of oxygen saturation goals in premature infants. *Arch Dis Child Fetal Neonatal Ed.* 2011;96(2):F93-8.
9. van der Eijk AC, Dankelman J, Schutte S, Simonsz HJ, Smit BJ. An observational study to quantify manual adjustments of the inspired oxygen fraction in extremely low birth weight infants. *Acta Paediatrica* 2012;101(3):e97–104.
10. McDermott RE, Mikulak RJ, Beauregard MR. *The basics of FMEA*. Portland: Productivity Press, 1996.
11. DeRosier J, Stalhandske E, Bagian JP, Nudell T. Using health care Failure Mode and Effect Analysis: the VA National Center for Patient Safety's prospective risk analysis system. *Joint Commission Journal on Quality and Patient Safety* 2002;28(5):248-67.
12. Habraken M, Van der Schaaf T, Leistikow I, Reijnders-Thijssen P. Prospective risk analysis of health care processes: a systematic evaluation of the use of HFMEA™ in Dutch health care. *Ergonomics* 2009;52(7): 809-19.
13. Van der Hoeff NWS. Theory and practice of in-hospital patient risk management Delft University of Technology, 2003.
14. Stark AR. Levels of neonatal care. *Pediatrics* 2004;114(5):1341-7.
15. Van der Hoeff NWS. www.patientveiligheid.org/handleidingen, 2011.
16. Ashley L, Armitage G, Neary M, Hollingsworth G. A practical guide to Failure Mode and Effects Analysis in health care: Making the most of the team and its meetings. *Joint Commission Journal on Quality and Patient Safety* 2010;36(8):351-58.
17. Chow LC, Wright KW, Sola A. Can changes in clinical practice decrease the incidence of severe retinopathy of prematurity in very low birth weight infants? *Pediatrics* 2003;111(2):339-45.
18. Wilkinson DJ, Andersen CC. Bedside algorithms for managing desaturation in ventilated preterm infants: A randomised crossover trial. *Neonatology* 2008;95(4):306-10.
19. Nghiem TH, Hagadorn JI, Terrin N, Syke S, MacKinnon B, Cole CH. Nurse opinions and pulse oximeter saturation target limits for preterm infants. *Pediatrics* 2008;121(5):e1039-46.
20. Armbruster J, Schmidt B, Poets CF, Bassler D. Nurses' compliance with alarm limits for pulse oximetry: qualitative study. *Journal of Perinatology* 2009;30:531-34.
21. Adachi W, Lodolce AE. Use of failure mode and effects analysis in improving the safety of iv drug administration. *American Journal of Health-System Pharmacy* 2005;62(9):917-20.
22. Apkon M, Leonard J, Probst L, DeLizio L, Vitale R. Design of a safer approach to intravenous drug infusions: failure mode effects analysis. *Quality and Safety in Health Care* 2004;13(4):265-71.
23. Cheng CH, Chou CJ, Wang PC, Lin HY, Kao CL, Su CT. Applying HFMEA to Prevent Chemotherapy Errors. *Journal of Medical Systems* 2010:1-9.

24. Esmail R, Cummings C, Dersch D, Duchscherer G, Glowa J, Liggett G. Using Healthcare Failure Mode and Effect Analysis tool to review the process of ordering and administrating potassium chloride and potassium phosphate. *Patient safety* 2005;8(Sp):73-80.
25. Moss J. Reducing errors during patient-controlled analgesia therapy through Failure Mode and Effects Analysis. *Joint Commission Journal on Quality and Patient Safety* 2010;36(8):359-64.
26. Kunac DL, Reith DM. Identification of priorities for medication safety in neonatal intensive care. *Drug Safety* 2005;28(3):251-61.
27. Askie L, Henderson-Smart DJ, Ko H. Cochrane review: Restricted versus liberal oxygen exposure for preventing morbidity and mortality in preterm or low birth weight infants. *Evidence-Based Child Health: A Cochrane Review Journal* 2010;5(1):371-413.
28. Ashley L, Armitage G. Failure Mode and Effects Analysis: An Empirical Comparison of Failure Mode Scoring Procedures. *Journal of Patient Safety* 2010;6(4):210-5.

Part V

Epilogue

Chapter 13

Summary and general discussion

Partly based on: Denise Rook & Johannes B. van Goudoever. Oxidative stress and Glutathione synthesis rates in early postnatal life. In: Studies on Perinatal & Prenatal Disorders. In press.

Due to ongoing developments and research, mortality and outcome of preterm infants have greatly improved over the last decades[1 2]. While the number of survivors is increasing and the gestational age (GA) of survivors is decreasing, morbidity after preterm birth is not decreasing. Therefore, reducing morbidity should also be a main objective of neonatal research. Neonatal diseases like bronchopulmonary dysplasia, intraventricular hemorrhage, retinopathy of prematurity and necrotizing enterocolitis continue to be a great problem in neonatology and greatly influence the outcome of preterm infants[1 2].

PART I: GENERAL INTRODUCTION

The pathogenesis of these diseases is still not fully unraveled, but seems to be multifactorial. It has been postulated, first by dr. Ola Saugstad, that oxidative stress plays a role in the pathogenesis of all of these diseases. As described in **Chapter 1**, oxidative stress is the resultant from an imbalance between antioxidant defense mechanisms and the formation of reactive oxygen species (ROS). Preterm infants are said to be at increased risk of oxidative stress due to immaturity of their antioxidant defenses and increased production of ROS. To improve the long-term outcome of preterm infants, research towards reduction of damage by oxidative stress is important and should be focused both on increasing the antioxidant defenses and reduction of oxidative stress.

Clinical outcome, i.e. 'oxygen radical diseases of neonatology', is the main outcome when studying the effects of an intervention on oxidative stress in preterm infants. However, indicators of oxidative stress are also important. To assess the level of oxidative stress, we measured biomarkers of oxidative stress and glutathione (GSH) kinetics, which is the main intracellular antioxidant. Markers of oxidative stress were measured in urine, which is readily available without harming preterm infants. GSH kinetics were determined in erythrocytes. Since research in preterm infants is limited by small blood volumes, it is therefore important to reliably measure GSH enrichments and concentrations in small samples. **Chapter 2** describes the development of the simultaneous analysis of both GSH and its precursor glycine using liquid chromatography-isotope ratio mass spectrometry (LC/IRMS), thereby reducing the blood volume needed to measure GSH kinetics.

PART II: OXIDATIVE STRESS AT BIRTH

The first focus of this thesis was to reduce the oxidative insult as a result of oxygen administration during the resuscitation after birth of preterm infants, while warranting a safe transition to extrauterine life (**Chapters 3-5**). Due to immature lungs, preterm infants often require some sort of respiratory support with supplemental oxygen after birth in order to reach adequate

oxygenation. However, oxygen during resuscitation, even when only administered for a few minutes, can have long lasting detrimental effects in term infants[3]. In a meta-analysis including 10 studies and over 2000 infants, the risk of neonatal mortality was reduced after resuscitation with 21% oxygen compared to 100% oxygen (relative risk 0.69, 95% confidence interval 0.54-0.88)[3]. This reduction was even more striking when including only randomized studies in the meta-analysis. There was also a trend toward a decrease in the risk of hypoxic ischemic encephalopathy after resuscitation with 21% oxygen compared to 100% oxygen. Although recent resuscitation guidelines advise to start resuscitation of preterm infants with lower oxygen concentrations, the optimal fraction of inspired oxygen (FiO_2) for preterm infants during resuscitation is still undetermined.

Initial FiO_2 during the resuscitation of preterm infants

Previous studies on the initial fraction of inspired oxygen (FiO_2) during the resuscitation of preterm infants compared the use of low FiO_2, i.e. room air or 30% oxygen, with either 90% or 100% oxygen[4-7]. In these studies, FiO_2 in the low-oxygen group had to be increased to reach appropriate oxygenation, while the FiO_2 in the high-oxygen group was often decreased. Resuscitation of preterm infants with an initial FiO_2 of 30% resulted in decreased oxidative stress markers and a decreased risk of bronchopulmonary dysplasia (BPD) compared to an initial FiO_2 of 90%[6]. Based on these studies, we concluded that initiating the resuscitation of preterm infants with room air was too low, while starting with 90% FiO_2 was too high. As stated by Augusto Sola: "Errors have been generalizing guidelines for FiO_2 in delivered gas, focusing on FiO_2 extremes (0.21 and 1.0) when there are 79 other options"[8 9]. Therefore, to assess the safety and efficacy of starting resuscitation of preterm infants with low oxygen concentrations, the next objective was to compare 30% oxygen with an intermediate FiO_2.

For this aim, we performed a double-blind, randomized controlled trial comparing an initial FiO_2 of 30% with an initial FiO_2 of 65% for the resuscitation of very preterm infants (**Chapters 3 and 4**). In total, we included 193 preterm infants with a GA below 32 weeks who were randomized to start resuscitation with either 30% or 65% oxygen for which the physician was blinded. In this study, we demonstrated that resuscitation of preterm infants with 30% oxygen is as safe as resuscitation with 65% oxygen, but does not offer benefits with regard to survival without BPD. The amount of oxygen administered in the high-oxygen group was significantly higher during the first five minutes. Since preterm infants are difficult to ventilate in the immediate postnatal phase due to immaturity of the lungs, oxygen supplementation will not warrant immediate effect and might result in overexposure of the lung to oxygen. In our study, the higher oxygen load actually resulted in higher oxygen saturations, indicating that the oxygen administered diffused into the blood stream. In addition, preterm infants lack sufficient (pulmonary) antioxidant defences to overcome sudden exposure to oxygen[10 11], making them more susceptible for oxidative (lung) damage. Taken together, we conclude that resuscitation of preterm infants with a GA below 32 weeks is preferably done with an initial FiO_2 of 30%, rather than with 65%.

As soon as the lungs are aerated and oxygen saturation rises, individual adjustment of the FiO_2 is needed based on oxygen saturation measured by pulse oximetry (SpO_2).

Although we could not demonstrate differences in short-term clinical outcome and in oxidative stress, long-term outcome of these infants will unravel if there are subtle differences due to different exposure to oxygen, that did not manifest in the neonatal period. As part of the randomized study, we will determine the neurodevelopmental outcome at 2 years of age. The long-term follow up is described in **Chapter 5**. Currently, the follow up is still ongoing and deblinding will be performed after all infants have been tested for neurodevelopmental outcome at 2 years of corrected age.

FiO_2 adjustment during the resuscitation of preterm infants

After the initial phase of the resuscitation of preterm infants, subsequent adjustment of the FiO_2 should be based on individual SpO_2 measurements. In 2010, the resuscitation guideline of the European Resuscitation Council (ERC) introduced SpO_2 targets for the first 10 minutes after birth[12]. These SpO_2 targets are based on a study by Dawson et al., where normative values of heart rate and SpO_2 were obtained in 468 healthy, mostly term infants who did not require respiratory support during resuscitation[13]. It is debatable whether these SpO_2 targets are appropriate for preterm infants. In the study by Dawson et al., preterm infants who did not require respiratory support during resuscitation after birth showed a slower rise in SpO_2 after birth[13]. It can be discussed what the appropriate SpO_2 targets are for preterm infants; those of healthy term infants or those of preterm infants who do not require respiratory support. To date, the current ERC guidelines are however the best available evidence and these SpO_2 targets should be used to guide FiO_2 administration during resuscitation.

Currently, to reach and maintain the SpO_2 targets, the fraction of inspired oxygen (FiO_2) is adjusted manually but the manual control of the SpO_2 is known to be difficult, as demonstrated by reported time spend outside the target range of approximately 50% in neonatal intensive care units (NICU)[14-17]. During resuscitation, the unknown maturation of the infants lungs, the need for the SpO_2 to rise and the complex proceedings of the resuscitation make controlling the saturation even more difficult. The guidelines do not provide any advice on how and when to adjust the FiO_2 and although some strategies have been published, they have yet to be proven effective[18]. As shown in **Chapter 6**, following the SpO_2 targets has proven to be difficult in current clinical practice. In this observational study, large deviations from the SpO_2 targets were observed during the resuscitation of 78 very preterm infants. During the first minutes of resuscitation the deviations were probably caused by an inability to control the SpO_2 due to absent or unreliable SpO_2 measurement in the immediate postnatal phase, difficulties in appropriate aeration of the immature lungs or suboptimal ventilation. The single value targets from the ERC can never be met precisely and it seems therefore preferable to define an acceptable target range and evaluate this in clinical practice.

Conclusion and future perspectives

The initial FiO_2 for the resuscitation of preterm infants has been studied in multiple trials with different initial FiO_2, different GA of included infants and different SpO_2 targets for subsequent adjustments[4-7]. From these studies, it can be concluded that resuscitation should be initiated with low oxygen concentrations. Our study demonstrates that an initial supplementation of preterm infants with 30% oxygen during fetal-to-neonatal transition is as safe as 65% oxygen and results in reduced oxygen exposure during resuscitation, but does not offer benefits with respect to short-term clinical outcome and oxidative stress. Currently, we are awaiting long-term neurodevelopmental follow up.

We acknowledge, however, a potentially important limitation of our study design. As parental informed consent was needed prenatally, we selected cases with expected premature birth as is seen in preeclampsia, intrauterine growth retardation and chorioamnionitis. We did not include the cases with unexpected acute premature labor, in whom there was no opportunity for administration of prenatal steroids. Future studies should include a waiver of consent in order to also include infants without prenatal steroids. Prenatal steroids are known to positively influence antioxidant defense in preterm infants and therefor untreated infants might be at increased risk for overexposure to oxygen[19]. In addition, combining data of all completed and ongoing studies in a meta-analysis might reveal subtle effects, since large numbers are needed to demonstrate differences in less common outcomes, such as mortality.

After the initial FiO_2, subsequent adjustments should be based on individual SpO_2 measurements. To date, no randomized studies have been performed on the appropriate SpO_2 target range during the resuscitation of preterm infants. To compare different SpO_2 target regimes, it is important that current clinical practice is able to control the SpO_2 adequately, which is a challenging task. New technological developments might aid in SpO_2 control. By displaying actual SpO_2 and the target together on one screen, deviation from the target and the trend of the actual SpO_2 are better interpreted and this might contribute to improved control of the SpO_2. The use of a respiratory function monitor, such as the one developed by Schmölzer et al [20], might contribute to controlling the infants oxygenation by supplying feedback on ventilation status. Closed loop SpO_2 control is available for use in a NICU setting (CliO2, CareFusion, San Diego, USA), and similar technology might be beneficial during resuscitation.

PART III: NUTRITIONAL MODULATION OF OXIDATIVE STRESS

Another way of preventing oxidative stress is to enhance antioxidant defense, which might be achieved via nutritional modulation. Protein synthesis, like the synthesis of GSH, is dependent on adequate substrate, i.e. amino acids (AA). In addition, a positive energy balance is important for protein synthesis, since protein synthesis is an energy demanding process. Non-protein

energy deficits are, however, still common in premature infants during the first week of life[21]. Optimizing early nutrition by increasing AA intake and early administration of lipids might improve antioxidant defenses by increased GSH synthesis.

Amino acids

Frans te Braake et al. previously showed that early administration of 2.4 g/(kg•d) AAs to preterm infants resulted in increased concentration of GSH on the second day of life, without a concomitant rise in the fractional synthesis rate of GSH (FSR_{GSH})[22]. In follow up of this study, we demonstrate in **Chapter 7** significantly lower GSH concentrations within a few hours after birth as compared to the second day of life in infants receiving 2.4 g/(kg•d) AAs. Also in this study, FSR_{GSH} was not increased. These data suggest that the increased GSH concentration after initial low concentrations after birth could be the result of decreased GSH consumption upon AA administration. Possible explanations for this might be that AAs, including methionine and cysteine, can serve as antioxidants themselves[23] or that increased availability of AAs up-regulates synthesis of other antioxidants like albumin synthesis[24]. Besides antioxidant properties, GSH also functions as a cysteine reservoir[25 26] and it might be possible that increased availability of cysteine reduces breakdown of GSH to generate free cysteine. As demonstrated in **Chapter 8**, increasing AA administration even further to 3.6 g/(kg•d) did not have an additional effect on either GSH availability or synthesis rate.

Lipids

Lipids are an essential component of the parenteral nutrition of preterm infants, because they supply the essential fatty acids and are a source of high calories. Since lipids become the main source of energy within hours after administration[27], early lipid administration may result in protein sparing. In **Chapter 8**, we demonstrated that increasing the energy balance via lipid administration form birth onwards did not result in increased GSH synthesis. As lipids are vulnerable for lipid peroxidation, it might be possible that early introduction of parenteral lipids could contribute to increased oxidative stress in preterm infants. Total isofurans and neurofurans were increased on postnatal day 2 upon early lipid administration. However, these differences disappeared in the consecutive days when all groups received lipids and other oxidative stress markers were not different between groups. Furthermore, early initiation of lipids did not results in differences in short-term clinical outcome (Vlaardingerbroek et al., *submitted*). Therefore, it seems that lipid administration from birth onwards does not increase oxidative stress compared to initiation of lipids later, but long-term follow-up will determine whether the transient increase of isofurans and neurofurans upon early initiation of lipids is indeed clinically irrelevant.

Besides the timing of parenteral lipid administration, we also studied the effect of the type of lipid emulsion administered. Traditional lipid emulsions are mainly manufactured from soybean oil and are very rich in ω-6 polyunsaturated fatty acids (PUFAs), which are highly

susceptible for lipid peroxidation resulting in increased oxidative stress[28-30]. A multicomponent lipid emulsion, containing soya bean oil, medium-chain triglycerides, olive oil and fish oil with increased added α-tocopherol, might positively influence oxidative stress due to a more balanced ω-3:ω-6 fatty acid ratio and the added α-tocopherol. However, literature comparing the effect of a multicomponent lipid emulsion with a pure soybean oil emulsion on oxidative stress is conflicting[31-35]. Also, parenteral lipids were supplied either with low doses form birth onwards or lipids were not started directly from birth onwards in most of these studies. Therefore, we performed a double-blind randomized study comparing a multicomponent lipid with a pure soybean oil emulsion with a starting dose of 2 g/(kg•d) directly after birth and 3 g/(kg•d) on the following days (**Chapter 9**). GSH concentration and synthesis rate were not altered following administration of different lipids emulsions from birth onward in preterm infants. Besides a decrease in isofurans (indicative of a reduced arachidonic acid peroxidation), all other markers of oxidative stress were not reduced in infants receiving a multicomponent lipid emulsion. Although the association of a reduction in isofurans seems to suggest an interplay between lipid peroxidation and oxygen rather than an effect of sole lipid peroxidation, the other markers were not affected and type of lipid does not affect neonatal morbidities[36], the implication of differences in isofurans is unknown. Taken together, there does not seem to be a clear reduction of oxidative stress upon administration of the multicomponent lipid emulsion compared to a pure soybean oil emulsion.

Conclusion and future perspectives

Initiation of AAs from birth onwards resulted in increased GSH concentration, probably caused by decreased GSH consumption. Extracellular amino acid substrate availability suppresses the cellular glutathione loss through oxidation, thereby facilitating the preservation of cellular glutathione content[37]. Consequently, increased AA availability limits GSH recycling and the need for de-novo synthesis. However, there appears to be a limit to this effect on GSH, since high dose AA administration did not have an additive effect.

Increasing energy intake via intravenous lipids also did not result in increased GSH synthesis. However and fortunately, timing and type of lipid emulsion supplied as part of parenteral nutrition to preterm infants does not seem to influence oxidative stress significantly. Probably, oxidative stress is already so highly increased in preterm infants that parenteral lipids do not have a detectable effect. However, long-term follow up will reveal whether the differences in isofurans and neurofurans are of clinical relevance.

In conclusion, nutritional modulation of GSH synthesis in preterm infants seems to warrant only minimal effects. Increased AAs and/or lipids do not increase GSH synthesis rate. Maximum synthetic capacity caused by immaturity of the enzymatic apparatus in preterm infants is not the (sole) explanation for this, since preterm infants seem to be capable of synthesizing GSH (discussed elsewhere in this chapter). Shortage of substrate should have been intercepted by increased AA administration, but the composition of the AA might have been suboptimal.

Protein synthesis is dependent on availability of all precursor AAs and the rate of protein synthesis is thus determined by the first limiting AA.

Generally, the AA content of human milk should be adequate for term infants and, therefore, most infant formulas have a composition comparable to human milk. Specific requirements for preterm infants might be different, since preterm infants have to experience a postnatal growth in the extrauterine environment equivalent to intrauterine growth. Cysteine was always considered to be conditionally essential in preterm infants, but shortage of cysteine also does not seem to be the main limiting factor in preterm infants (discussed elsewhere in this chapter). Currently, studies are undertaken to establish requirements of the individual AAs in preterm infants and future studies should investigate the effect of other AA compositions with these requirements on GSH synthesis. Another course of future research on nutritional modulation of oxidative stress is the supplementation of vitamins and other antioxidants in preterm infants. However, to date, results are inconclusive and the search for the holy grail continues.

PART VI: OTHER MODULATORS OF OXIDATIVE STRESS

Effect of neonatal sepsis on oxidative stress

Many preterm infants will develop nosocomial sepsis during the first weeks of life, with reported incidences of 21 to 36%[2 38-40]. Despite major improvements in neonatal care, sepsis remains an important cause of morbidity and mortality in preterm infants. Amongst others, sepsis in preterm infants is associated with severe adverse neurodevelopmental outcome at 2 years of age[41 42]. The relation between neonatal sepsis and the adverse outcome is likely to be multifactorial, but oxidative stress is thought to be one of the major contributors[41 43]. During sepsis, ROS formation is increased as part of the defensive response against pathogens through the stimulation of pro-inflammatory cytokine release, the formation of chemotactic factors and neutrophil recruitment [44-47]. In both adults and children, overstimulation of ROS production or an insufficient antioxidant capacity is associated with increased morbidity and mortality[48-50]. A compromised antioxidant defense makes preterm infants more susceptible to oxidative stress[51 52], and the increased ROS formation during sepsis might be an etiological factor to the detrimental long-term outcome in preterm infants after neonatal sepsis. In **Chapter 10**, however, we demonstrated that nosocomial sepsis in premature infants did not result in increased oxidative stress and therefore there was no up-regulation of GSH synthesis.

Our study design might have been suboptimal. We designed the study to detect differences in GSH kinetics and the study was therefore not powered to detect differences in oxidative stress markers. Nowadays, less invasive methods to measure oxidative stress markers, i.e. urine, are available. This allows future studies to include more preterm infants and also to include 'healthy' preterm infants as controls. Our data do not explain the association between nosocomial neonatal sepsis in preterm infants and adverse long-term outcome. It would be of great

interest to further explore other mechanisms, such as the production of pro-inflammatory cytokines which are known to be neurotoxic and altered cerebral blood flow through circulatory insufficiency.

Determinants of GSH synthesis

Shorter gestation, lower birth weight (BW) and male gender are associated with poorer outcome. This effect might be partly mediated by differences in antioxidant capacity. Since oxidative stress is associated with both morbidity and mortality in preterm infants, we hypothesized that gender, GA and BW would affect GSH kinetics. However, differences in outcome related to GA, BW and gender seem to be unexplained by differences in GSH synthesis rates (**Chapter 11**).

Interestingly, the GSH-GSSG ratio was slightly lower in females than in males, which is in contrast with previous reports on gender differences in oxidative stress. This should be further investigated with, amongst others, measurement of multiple parameters of oxidative stress simultaneously.

The process of oxygen supplementation

Another important factor leading to increased oxidative stress in preterm infants is the oxygen supplementation during their stay on the NICU. Due to immature lungs, supplemental oxygen therapy is often needed in the first days to weeks of life to maintain adequate oxygenation. Unfortunately, supplemental oxygen therapy has a very narrow therapeutic range, since both hypoxia and hyperoxia may have severe consequences for the outcome of these infants[53]. Currently, FiO_2 is adjusted manually to maintain SpO_2 within the SpO_2 target range. As stated by Alexander Pope: "To err is human" (*An Essay on Criticism*, 1711). Therefore, the process of manually controlling SpO_2 might be subjected to flaws. This is underscored by the Neonatology System for Analysis and Feedback on medical Events (NEOSAFE), where (near-) accidents are reported by NICU personnel on an anonymous and voluntary basis. In 2010, only 6% of the reports on the NICU of the Erasmus Medical Center-Sophia Children's Hospital in Rotterdam were related to 'supplemental oxygen therapy'. However, these reports were responsible for 90% of the (near-)accidents that were categorized as 'most risky'. Therefore, we performed a Failure Mode and Effects Analysis (FMEA)' to evaluate hazards in the process of supplemental oxygen therapy in very preterm infants hospitalized on the NICU, and to provide recommendations for improvement (**Chapter 12**).

The top ten hazards could all be categorized to three main topics: incorrect adjustment of the FiO_2, incorrect alarm limits for SpO_2 and incorrect pulse-oximetry alarm limits on patient monitors for temporary use. The FMEA culminated in recommendations in both educational and technical directions. These included suggestions for (changes in) protocols on alarm limits and manual FiO_2 adjustments, education of NICU staff on hazards of supplemental oxygen, and technical improvements in respiratory devices and patient monitors.

GENERAL DISCUSSION

Oxidative stress and "fetal origins" of adult diseases

Fetal development takes place in a relative hypoxic environment, with the partial pressure of oxygen (pO_2) in the fetus rarely above 4 kPa[54]. As soon as infants start breathing, the pO_2 rises sharply. This sudden increase in pO_2 at birth is essential for cardiopulmonary adaptation at birth, which includes a decrease of pulmonary vascular resistance and vascular remodeling, like the closure of the ductus arteriosus[55 56]. Hence, a certain amount of oxygen is essential for an adequate transition at birth. To achieve a normal oxygenation, preterm infants often require additional oxygen during resuscitation after birth. However, oxygen exposure goes hand in hand with the formation of reactive oxygen species (ROS), while preterm infants are already highly susceptible to oxidative damage due to their immature antioxidant defenses. So, there seems to be a delicate balance between hypoxia and hyperoxia during the initial phase after birth, which might have long-term consequences.

Fetal origins of diseases have been studied extensively, mainly focusing on growth and nutrition. Dr. Barker and Dr. Hales (1992) were the first to retrospectively observe that poor fetal growth, and thus fetal malnutrition, is associated with impaired glucose tolerance, the metabolic syndrome and cardiovascular risk in adulthoods. Since then, many others studied this association, both epidemiologically and biochemically, and this association became known as the 'Barker hypothesis'. However, the underlying mechanisms remain poorly understood. Major hypotheses on this developmental programming include the 'thrifty phenotype', postnatal accelerated or catch-up growth, glucocorticoid effects and epigenetic changes[57]. Recently, it has been hypothesized that oxidative stress might also be involved in this fetal programming for diseases in later life[57-60].

There are several mechanisms how ROS and/or oxidative stress might be implicated in fetal programming. First, ROS and the redox balance play an important role as regulatory mediators in signaling processes. This is important for the control of gene expression during physiological processes like programmed cell death in fetal development, the physiological response to pathogens and maintaining vascular tone[61 62]. However, increased ROS levels change the normal redox balance with subsequent alterations of gene expression[63]. Second, ROS are highly reactive molecules and will react with other molecules in order to stabilize themselves. This includes direct damage to nucleotides, which could result in permanent DNA mutations when not repaired appropriately. Furthermore, ROS can cause epigenetic changes, like modification of methylation or telomere length, leading to permanent changes in gene transcription and expression. Finally, oxygen sensing might be altered under different conditions of oxidative stress. Specialized cells, like cells of the carotid body, neuroepithelial bodies in the lungs and smooth-muscle cells of the pulmonary arteries and the ductus arteriosus, sense the local oxygen tension and constitute a specialized homeostatic oxygen-sensing system[64]. The transcription of hypoxia-inducible factor-1α (HIF-1α) is oxygen dependent and is an integral part

of this oxygen-sensing system[54 62 65]. HIF-1a is an is activated during hypoxia and transcribes a large number of genes which defend the organism against hypoxia, including genes related to angiogenesis, erythropoiesis and glucose uptake resulting in reduced oxygen consumption and increased oxygen delivery. In normoxia and hyperoxia, HIF-1a is turned off and degraded. Adaptation of the human body encompasses a whole array of changes and exposure to hypoxia or hyperoxia during this adaptation might result in altered oxygen sensing, with an adjustment of the normoxia set point so that cells perceive hyperoxia under otherwise normal oxygen concentrations[54].

Especially preterm infants are subjected to increased oxidative stress, both during fetal development and the neonatal period. Many conditions associated with preterm birth, such as pre-eclampsia and chorioamnionitis, have also been associated with oxidative stress[66 67]. After birth, preterm infants are also subjected to increased levels of oxidative stress during a period where preterm infants experience equivalent intrauterine development stage in an oxygen-rich environment postnatally[58]. Subsequently, this increased oxidative stress during this 'critical window' might predispose for diseases in later life. This stresses the importance of long-term follow-up and meta-analysis of smaller studies, to establish the effects of oxygen in the early phase of premature life on health and functioning in adult life.

Glutathione kinetics

In this thesis, GSH kinetics were used as a marker for oxidative stress. GSH is the main intracellular non-enzymatic antioxidant and kinetic studies should contribute to a better understanding into antioxidant response to an oxidative insult. In the clinical studies presented in this thesis, we measured GSH synthesis in erythrocytes. Besides being readily accessible as opposed to other tissues, erythrocytes are also suggested to function as antioxidant defense by being a physiological source of GSH and by taking up ROS[51 68]. Giustarini et al. (2007) provided strong evidence for a role of erythrocytes as GSH donor for other tissues[68]. In this manner, erythrocytes can provide protection against oxidative injury to other tissues, such as the lungs, by supplying them with intracellular antioxidant[51 69-72]. Furthermore, membranes of erythrocytes are permeable to superoxide and hydrogen peroxide. By taking up these ROS, erythrocytes are also able to protect other tissues against oxidative damage[73 74]. Taken together, erythrocytes seem to be a good representative of systemic oxidative stress.

In the studies presented in this thesis, erythrocytic fractional synthesis rate (FSR) was not upregulated upon any intervention (i.e. oxygen administration, nutritional interventions or during nosocomial sepsis), which raises the question why preterm infants exhibit such a stable FSR. Immaturity of the enzymatic apparatus might be an obvious explanation. Glutamate-cysteine ligase - the rate-limiting enzyme in GSH synthesis - is present and active in preterm infants[75]. However, literature about cystathionase, which converts homocysteine to L-cysteine, is conflicting[76-79]. It has been suggested that cystathionase is not fully matured in preterm infants, making cysteine a conditionally essential AA in preterm infants. On the other hand,

stable isotope studies have shown that conversion of homocysteine to cysteine is not hampered in preterm infants[80 81]. Moreover, biosynthesis of glutathione has proven to be active in leukocytes from preterm infants[82] and is not dependent on gestational age (**Chapter 11**). As discussed previously in **Chapter 11**, it would be very interesting to investigate whether genetic differences, like polymorphisms, in the GSH synthesis apparatus might alter GSH synthesis and its response to oxidative stress. Polymorphisms in other antioxidant enzymes have already been associated with perinatal mortality and neonatal morbidity[83-86].

Another explanation for the absence of up-regulation of GSH synthesis in response to the postnatal decrease of GSH synthesis could be lack of sufficient substrate. Providing preterm infants with additional parenteral AAs does however not up-regulate GSH synthesis (**Chapters 7 and 8**). Cysteine is considered to be the rate-limiting substrate for glutathione synthesis. Although the capacity for cysteine synthesis is thus present in preterm infants, demands might very well exceed the capacity for de novo synthesis. Preterm infants could thus still be dependent on exogenous cysteine, while most neonatal AA solutions contain small amounts of cysteine or none at all. However, increased parenteral cysteine administration did not result in increased GSH synthesis[87]. Also in the our study, cysteine concentrations were not correlated with GSH synthesis (**Chapter 8**). Taken together, lack of cysteine, either by cystathionase deficiency or insufficient substrate, does not seem to be the main determinant of GSH synthesis in preterm infants. As discussed previously in this chapter, current AA solutions might not be optimal for preterm infants and protein synthesis is dependent of availability of all precursor AAs. Studies on the requirements of the individual AAs in preterm infants should reveal whether current AA solutions are sufficient for the needs of preterm infants.

Finally, preterm infants are shown to have a higher recycling of GSSG into GSH in erythrocytes compared with adults[88]. This more efficient recycling could decrease the need for de novo GSH synthesis, since there is relatively more effective GSH. It can, therefore, be questioned whether GSH synthesis rate alone is a suitable marker to study oxidative stress in preterm infants. Simultaneous measurement of all players of the GSH antioxidant defense system, namely GSH synthesis, the ratio of GSH and its oxidized GSSG, and the enzymes involved in GSH synthesis and recycling, should provide a better insight into GSH in preterm infants.

In conclusion, preterm infants seem to be able to up-regulate GSH synthesis. The stimuli to do so, however, are unclear.

CLOSING REMARKS

As repeatedly stressed in this thesis, increased oxidative stress can have detrimental consequences, especially in preterm infants who are experiencing equivalent intrauterine development stage in an oxygen-rich environment postnatally. However, it is important to remember that oxidative stress also has a physiological function as in cardiopulmonary adaptation at

birth, the defense against microorganisms and in inter- and intracellular signaling. So, there seems to be a delicate balance between pathologic and physiologic oxidative stress.

Although we could not demonstrate differences in oxidative stress upon oxygen supplementation or differences in antioxidant defense upon nutritional interventions, it is evident that oxidative stress plays an important role in neonatal morbidity and mortality. Clearly, the mechanisms of oxidative stress and antioxidant defense are complex and warrant further research. We suggest that future research on oxidative stress in preterm infants should focus on finding more sensitive biomarkers of oxidative stress, genetic polymorphisms and the adaptive mechanisms.

REFERENCES

1. Incidence of and Risk Factors for Neonatal Morbidity after Active Perinatal Care: Extremely Preterm Infants Study in Sweden (EXPRESS). *Acta Paediatr* 2010;99(7):978-92.
2. Stoll BJ, Hansen NI, Bell EF, Shankaran S, Laptook AR, Walsh MC, et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics* 2010;126(3):443-56.
3. Saugstad OD, Ramji S, Soll RF, Vento M. Resuscitation of Newborn Infants with 21% or 100% Oxygen: An Updated Systematic Review and Meta-Analysis. *Neonatology* 2008;94(3):176-82.
4. Dawson JA, Kamlin CO, Wong C, Te Pas A, O'Donnell CP, Donath SM, et al. Oxygen saturation and heart rate during delivery room resuscitation of infants <30 weeks gestation with air or 100% oxygen. *Archives of disease in childhood. Fetal and neonatal edition* 2008;94(2):F87-91.
5. Escrig R, Arruza L, Izquierdo I, Villar G, Saenz P, Gimeno A, et al. Achievement of targeted saturation values in extremely low gestational age neonates resuscitated with low or high oxygen concentrations: a prospective, randomized trial. *Pediatrics* 2008;121(5):875-81.
6. Vento M, Moro M, Escrig R, Arruza L, Villar G, Izquierdo I, et al. Preterm Resuscitation With Low Oxygen Causes Less Oxidative Stress, Inflammation, and Chronic Lung Disease. *Pediatrics* 2009;124(3):e439-49.
7. Wang CL, Anderson C, Leone TA, Rich W, Govindaswami B, Finer NN. Resuscitation of preterm neonates by using room air or 100% oxygen. *Pediatrics* 2008;121(6):1083-9.
8. Sola A, Deulofeut R. Oxygen and oxygenation in the delivery room. *J Pediatr* 2006;148(4):564-5.
9. Sola A. Oxygen for the preterm newborn: one infant at a time. *Pediatrics* 2008;121(6):1257.
10. Frank L, Sosenko IR. Prenatal development of lung antioxidant enzymes in four species. *J Pediatr* 1987; 110(1):106-10.
11. Frank L, Sosenko IR. Failure of premature rabbits to increase antioxidant enzymes during hyperoxic exposure: increased susceptibility to pulmonary oxygen toxicity compared with term rabbits. *Pediatr Res* 1991;29(3):292-6.
12. European_Resuscitation_Council. European Resuscitation Council Guidelines for Resuscitation 2010. Resuscitation: Elsevier, 2010.
13. Dawson JA, Kamlin CO, Vento M, Wong C, Cole TJ, Donath SM, et al. Defining the Reference Range for Oxygen Saturation for Infants After Birth. *Pediatrics* 2010;125(6):e1340-7.
14. Bhutani VK, Taube JC, Antunes MJ, Delivoria Papadopoulos M. Adaptive control of inspired oxygen delivery to the neonate. *Pediatric pulmonology* 1992;14(2):110-17.
15. Automatic control of neonatal fractional inspired oxygen; 1991. IEEE.
16. van der Eijk AC, Dankelman J, Schutte S, Simonsz HJ, Smit BJ. An Observational Study to Quantify Manual Adjustments of the Inspired Oxygen Fraction in Extremely Low Birth Weight Infants. *Acta Paediatrica* 2011.
17. Hagadorn JI, Furey AM, Nghiem TH, Schmid CH, Phelps DL, Pillers DAM, et al. Achieved versus intended pulse oximeter saturation in infants born less than 28 weeks' gestation: the AVIOx study. *Pediatrics* 2006;118(4):1574.
18. Dawson JA, Vento M, Finer NN, Rich W, Saugstad OD, Morley CJ, et al. Managing Oxygen Therapy during Delivery Room Stabilization of Preterm Infants. *J Pediatr* 2011.
19. Vento M, Aguar M, Escobar JJ, Arduini A, Escrig R, Brugada M, et al. Antenatal Steroids and Antioxidant Enzyme Activity in Preterm Infants: Influence of Gender and Timing. *Antioxid Redox Signal* 2009.
20. Schmölzer G, Kamlin O, Dawson J, Te Pas A, Morley C, Davis P. Respiratory monitoring of neonatal resuscitation. *Archives of disease in childhood. Fetal and neonatal edition* 2010;95(4):F295.
21. Hulst JM, Goudoever JB, Zimmermann LJI, Hop WCJ, Albers MJIJ, Tibboel D, et al. The effect of cumulative energy and protein deficiency on anthropometric parameters in a pediatric ICU population. *Clinical Nutrition* 2004;23(6):1381-89.

22. Te Braake FW, Schierbeek H, de Groof K, Vermes A, Longini M, Buonocore G, et al. Glutathione synthesis rates after amino acid administration directly after birth in preterm infants. *Am J Clin Nutr* 2008; 88(2):333-9.
23. Manzella D, Grella R, Esposito K, Cacciapuoti F, Arciello A, Giugliano D, et al. Oral amino acid administration decreases oxidative stress and improves brachial reactivity in elderly individuals. *Am J Hypertens* 2005;18(6):858-63.
24. van den Akker CH, te Braake FW, Schierbeek H, Rietveld T, Wattimena DJ, Bunt JE, et al. Albumin synthesis in premature neonates is stimulated by parenterally administered amino acids during the first days of life. *Am J Clin Nutr* 2007;86(4):1003-8.
25. Stipanuk MH, Coloso RM, Garcia RA, Banks MF. Cysteine concentration regulates cysteine metabolism to glutathione, sulfate and taurine in rat hepatocytes. *The Journal of nutrition* 1992;122(3):420-7.
26. Stipanuk MH, Dominy JE, Jr., Lee JI, Coloso RM. Mammalian cysteine metabolism: new insights into regulation of cysteine metabolism. *The Journal of nutrition* 2006;136(6 Suppl):1652S-59S.
27. Vlaardingerbroek H, van Goudoever JB, van den Akker CH. Initial nutritional management of the preterm infant. *Early Hum Dev* 2009;85(11):691-5.
28. Alexander-North LS, North JA, Kiminyo KP, Buettner GR, Spector AA. Polyunsaturated fatty acids increase lipid radical formation induced by oxidant stress in endothelial cells. *Journal of lipid research* 1994;35(10):1773-85.
29. Helbock HJ, Motchnik PA, Ames BN. Toxic hydroperoxides in intravenous lipid emulsions used in preterm infants. *Pediatrics* 1993;91(1):83-7.
30. Laborie S, Lavoie JC, Chessex P. Increased urinary peroxides in newborn infants receiving parenteral nutrition exposed to light. *J Pediatr* 2000;136(5):628-32.
31. Skouroliakou M, Konstantinou D, Koutri K, Kakavelaki C, Stathopoulou M, Antoniadi M, et al. A double-blind, randomized clinical trial of the effect of omega-3 fatty acids on the oxidative stress of preterm neonates fed through parenteral nutrition. *Eur J Clin Nutr* 2010;64(9):940-7.
32. Koksal N, Kavurt AV, Cetinkaya M, Ozarda Y, Ozkan H. Comparison of lipid emulsions on antioxidant capacity in preterm infants receiving parenteral nutrition. *Pediatr Int* 2011;53(4):562-6.
33. Roggero P, Mosca F, Gianni ML, Orsi A, Amato O, Migliorisi E, et al. F2-isoprostanes and total radical-trapping antioxidant potential in preterm infants receiving parenteral lipid emulsions. *Nutrition* 2009.
34. Gobel Y, Koletzko B, Bohles HJ, Engelsberger I, Forget D, Le Brun A, et al. Parenteral fat emulsions based on olive and soybean oils: a randomized clinical trial in preterm infants. *Journal of pediatric gastroenterology and nutrition* 2003;37(2):161-7.
35. Webb AN, Hardy P, Peterkin M, Lee O, Shalley H, Croft KD, et al. Tolerability and safety of olive oil-based lipid emulsion in critically ill neonates: a blinded randomized trial. *Nutrition* 2008;24(11-12):1057-64.
36. Vlaardingerbroek H, Veldhorst MA, Spronk S, van den Akker CH, van Goudoever JB. Parenteral lipid administration to very-low-birth-weight infants--early introduction of lipids and use of new lipid emulsions: a systematic review and meta-analysis. *Am J Clin Nutr* 2012.
37. Yeung MY. Influence of early postnatal nutritional management on oxidative stress and antioxidant defence in extreme prematurity. *Acta Paediatr* 2006;95(2):153-63.
38. Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, et al. Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. *Pediatrics* 2002; 110(2 Pt 1):285-91.
39. van der Zwet WC, Kaiser AM, van Elburg RM, Berkhof J, Fetter WP, Parlevliet GA, et al. Nosocomial infections in a Dutch neonatal intensive care unit: surveillance study with definitions for infection specifically adapted for neonates. *J Hosp Infect* 2005;61(4):300-11.
40. Corpeleijn WE, Kouwenhoven SM, Paap MC, van Vliet I, Scheerder I, Muizer Y, et al. Intake of Own Mother's Milk during the First Days of Life Is Associated with Decreased Morbidity and Mortality in Very Low Birth Weight Infants during the First 60 Days of Life. *Neonatology* 2012;102(4):276-81.
41. Adams-Chapman I, Stoll BJ. Neonatal infection and long-term neurodevelopmental outcome in the preterm infant. *Current opinion in infectious diseases* 2006;19(3):290-7.

42. Stoll BJ, Hansen NI, Adams-Chapman I, Fanaroff AA, Hintz SR, Vohr B, et al. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. *Jama* 2004; 292(19):2357-65.
43. Volpe JJ. Neurobiology of periventricular leukomalacia in the premature infant. *Pediatr Res* 2001; 50(5):553-62.
44. Martins PS, Kallas EG, Neto MC, Dalboni MA, Blecher S, Salomao R. Upregulation of reactive oxygen species generation and phagocytosis, and increased apoptosis in human neutrophils during severe sepsis and septic shock. *Shock* 2003;20(3):208-12.
45. de Souza LF, Ritter C, Pens Gelain D, Andrades M, Bernard EA, Moreira JC, et al. Mitochondrial superoxide production is related to the control of cytokine release from peritoneal macrophage after antioxidant treatment in septic rats. *J Surg Res* 2007;141(2):252-6.
46. Hansson L, Seidegard J, Johansson L, Jeppsson B. Influence of glutathione metabolising enzymes in rats with gram-negative sepsis. *Eur J Surg* 2000;166(9):728-33.
47. Andrades M, Ritter C, Moreira JC, Dal-Pizzol F. Oxidative parameters differences during non-lethal and lethal sepsis development. *J Surg Res* 2005;125(1):68-72.
48. Victor VM, Rocha M, De la Fuente M. Immune cells: free radicals and antioxidants in sepsis. *Int Immunopharmacol* 2004;4(3):327-47.
49. Crimi E, Sica V, Slutsky AS, Zhang H, Williams-Ignarro S, Ignarro LJ, et al. Role of oxidative stress in experimental sepsis and multisystem organ dysfunction. *Free Radic Res* 2006;40(7):665-72.
50. von Dessauer B, Bongain J, Molina V, Quilodran J, Castillo R, Rodrigo R. Oxidative stress as a novel target in pediatric sepsis management. *J Crit Care* 2010.
51. Ahola T, Levonen AL, Fellman V, Lapatto R. Thiol metabolism in preterm infants during the first week of life. *Scand J Clin Lab Invest* 2004;64(7):649-58.
52. Davis JM, Auten RL. Maturation of the antioxidant system and the effects on preterm birth. *Semin Fetal Neonatal Med* 2010.
53. Finer N, Leone T. Oxygen Saturation Monitoring for the Preterm Infant: The Evidence basis for Current Practice. *Pediatric research* 2009;65(4):375-80.
54. Maltepe E, Saugstad OD. Oxygen in health and disease: Regulation of oxygen homeostasis - Clinical implications. *Pediatr Res* 2008;65(3):261-8.
55. Noori S, Wlodaver A, Gottipati V, McCoy M, Schultz D, Escobedo M. Transitional changes in cardiac and cerebral hemodynamics in term neonates at birth. *J Pediatr* 2012;160(6):943-8.
56. Noori S, Stavroudis TA, Seri I. Systemic and cerebral hemodynamics during the transitional period after premature birth. *Clinics in perinatology* 2009;36(4):723-36, v.
57. Luo ZC, Xiao L, Nuyt AM. Mechanisms of developmental programming of the metabolic syndrome and related disorders. *World journal of diabetes* 2010;1(3):89-98.
58. Luo ZC, Fraser WD, Julien P, Deal CL, Audibert F, Smith GN, et al. Tracing the origins of "fetal origins" of adult diseases: programming by oxidative stress? *Med Hypotheses* 2006;66(1):38-44.
59. Thompson LP, Al-Hasan Y. Impact of oxidative stress in fetal programming. *Journal of pregnancy* 2012; 2012:582748.
60. Wells PG, McCallum GP, Chen CS, Henderson JT, Lee CJ, Perstin J, et al. Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits, and cancer. *Toxicological sciences : an official journal of the Society of Toxicology* 2009;108(1):4-18.
61. Droge W. Free radicals in the physiological control of cell function. *Physiological reviews* 2002;82(1): 47-95.
62. Haddad JJ. Oxygen-sensing mechanisms and the regulation of redox-responsive transcription factors in development and pathophysiology. *Respiratory research* 2002;3:26.
63. Saugstad OD. Hyperoxia in the term newborn: more evidence is still needed for optimal oxygen therapy. *Acta Paediatr Suppl* 2012;101(464):34-8.
64. Weir EK, Lopez-Barneo J, Buckler KJ, Archer SL. Acute oxygen-sensing mechanisms. *The New England journal of medicine* 2005;353(19):2042-55.

65. Martin DS, Khosravi M, Grocott MP, Mythen MG. Concepts in hypoxia reborn. *Crit Care* 2010;14(4):315.
66. Raijmakers MT, Roes EM, Poston L, Steegers EA, Peters WH. The transient increase of oxidative stress during normal pregnancy is higher and persists after delivery in women with pre-eclampsia. *Eur J Obstet Gynecol Reprod Biol* 2008;138(1):39-44.
67. Cheah FC, Pillow JJ, Kramer BW, Polglase GR, Nitsos I, Newnham JP, et al. Airway inflammatory cell responses to intra-amniotic lipopolysaccharide in a sheep model of chorioamnionitis. *Am J Physiol Lung Cell Mol Physiol* 2009;296(3):L384-93.
68. Giustarini D, Milzani A, Dalle-Donne I, Rossi R. Red blood cells as a physiological source of glutathione for extracellular fluids. *Blood Cells Mol Dis* 2007;40(2):174-9.
69. Biolo G, Antonione R, De Cicco M. Glutathione metabolism in sepsis. *Crit Care Med* 2007;35(9 Suppl): S591-5.
70. Berggren M, Dawson J, Moldeus P. Glutathione biosynthesis in the isolated perfused rat lung: utilization of extracellular glutathione. *FEBS letters* 1984;176(1):189-92.
71. Clahsen PC, Moison RM, Holtzer CA, Berger HM. Recycling of glutathione during oxidative stress in erythrocytes of the newborn. *Pediatr Res* 1992;32(4):399-402.
72. Griffith OW, Meister A. Glutathione: interorgan translocation, turnover, and metabolism. *Proceedings of the National Academy of Sciences of the United States of America* 1979;76(11):5606-10.
73. Heffner JE, Repine JE. Pulmonary strategies of antioxidant defense. *The American review of respiratory disease* 1989;140(2):531-54.
74. van Asbeck BS, Hoidal J, Vercellotti GM, Schwartz BA, Moldow CF, Jacob HS. Protection against lethal hyperoxia by tracheal insufflation of erythrocytes: role of red cell glutathione. *Science* 1985;227(4688): 756-9.
75. Levonen AL, Lapatto R, Saksela M, Raivio KO. Expression of gamma-glutamylcysteine synthetase during development. *Pediatr Res* 2000;47(2):266-70.
76. Lestas AN, Rodeck CH. Normal glutathione content and some related enzyme activities in the fetal erythrocytes. *Br J Haematol* 1984;57(4):695-702.
77. Martin JA, Pereda J, Martinez-Lopez I, Escrig R, Miralles V, Pallardo FV, et al. Oxidative stress as a signal to up-regulate gamma-cystathionase in the fetal-to-neonatal transition in rats. *Cell Mol Biol (Noisy-legrand)* 2007;53 Suppl:OL1010-7.
78. Pallardo FV, Sastre J, Asensi M, Rodrigo F, Estrela JM, Vina J. Physiological changes in glutathione metabolism in foetal and newborn rat liver. *Biochem J* 1991;274 (Pt 3):891-3.
79. Vina J, Vento M, Garcia-Sala F, Puertes IR, Gasco E, Sastre J, et al. L-cysteine and glutathione metabolism are impaired in premature infants due to cystathionase deficiency. *Am J Clin Nutr* 1995;61(5): 1067-9.
80. Riedijk MA, van Beek RH, Voortman G, de Bie HM, Dassel AC, van Goudoever JB. Cysteine: a conditionally essential amino acid in low-birth-weight preterm infants? *Am J Clin Nutr* 2007;86(4):1120-5.
81. Thomas B, Gruca LL, Bennett C, Parimi PS, Hanson RW, Kalhan SC. Metabolism of methionine in the newborn infant: response to the parenteral and enteral administration of nutrients. *Pediatr Res* 2008; 64(4):381-6.
82. Lavoie JC, Chessex P. Development of glutathione synthesis and gamma-glutamyltranspeptidase activities in tissues from newborn infants. *Free radical biology & medicine* 1998;24(6):994-1001.
83. Raijmakers MT, de Galan-Roosen TE, Schilders GW, Merkus JM, Steegers EA, Peters WH. The Tyr113His polymorphism in exon 3 of the microsomal epoxide hydrolase gene is a risk factor for perinatal mortality. *Acta obstetricia et gynecologica Scandinavica* 2004;83(11):1056-60.
84. Poggi C, Giusti B, Vestri A, Pasquini E, Abbate R, Dani C. Genetic polymorphisms of antioxidant enzymes in preterm infants. *J Matern Fetal Neonatal Med* 2012;25 Suppl 4:131-4.
85. Manar MH, Brown MR, Gauthier TW, Brown LA. Association of glutathione-S-transferase-P1 (GST-P1) polymorphisms with bronchopulmonary dysplasia. *J Perinatol* 2004;24(1):30-5.

86. Oretti C, Marino S, Mosca F, Colnaghi MR, De Iudicibus S, Drigo I, et al. Glutathione-S-transferase-P1 I105V polymorphism and response to antenatal betamethasone in the prevention of respiratory distress syndrome. *European journal of clinical pharmacology* 2009;65(5):483-91.
87. Te Braake FW, Schierbeek H, Vermes A, Huijmans JG, van Goudoever JB. High-dose cysteine administration does not increase synthesis of the antioxidant glutathione preterm infants. *Pediatrics* 2009; 124(5):e978-84.
88. Frosali S, Di Simplicio P, Perrone S, Di Giuseppe D, Longini M, Tanganelli D, et al. Glutathione recycling and antioxidant enzyme activities in erythrocytes of term and preterm newborns at birth. *Biol Neonate* 2004;85(3):188-94.

Chapter 14

Nederlandse samenvatting

Door wetenschappelijk onderzoek en verbetering van de zorg zijn de sterftecijfers en de uitkomst van premature pasgeborenen, dit zijn kinderen geboren na een zwangerschapsduur korter dan 37 weken, in de afgelopen decennia sterk verbeterd. Hoewel de overleving van premature pasgeborenen verbetert en ook pasgeborenen met een steeds kortere zwangerschapsduur overleven, neemt de ziektelast, ook wel morbiditeit genoemd, na vroeggeboorte niet af. Het verminderen van de morbiditeit is dan ook een belangrijk doel van onderzoek bij pasgeborenen. Neonatale aandoeningen zoals bronchopulmonale dysplasie (chronische longaandoening), intraventriculaire bloeding (hersenbloeding), retinopathie van prematuriteit (afwijking van het netvlies van het oog) en necrotiserende enterocolitis (ernstige darmziekte) blijven een groot probleem in de neonatale zorg en zijn van grote invloed op de uitkomst van premature pasgeborenen.

DEEL 1: INTRODUCTIE

De oorzaak van bovengenoemde neonatale aandoeningen is nog steeds niet volledig ontrafeld, maar lijkt multifactorieel te zijn. In de jaren 80, stelde Dr. Ola Saugstad de hypothese dat oxidatieve stress een rol speelt in de pathogenese van al deze ziekten en noemde deze aandoeningen 'zuurstofradicale ziekten van de neonatologie'. Zoals beschreven in **Hoofdstuk 1**, is oxidatieve stress het gevolg van een disbalans tussen antioxidanten en zuurstofradicalen. Hierbij worden zuurstofradicalen onvoldoende geneutraliseerd door antioxidanten, waardoor zij lichaamscellen en -weefsels kunnen beschadigen. Als gevolg van onrijpheid en tekort aan antioxidanten enerzijds en een toegenomen productie van zuurstofradicalen anderzijds, hebben premature pasgeborenen een verhoogd risico op het ontstaan van oxidatieve stress. Om de morbiditeit van premature pasgeborenen te verminderen, is onderzoek naar oxidatieve stress van groot belang en dient dit gericht te zijn op zowel het verbeteren van de antioxidanten van premature pasgeborenen als op het verminderen van vorming van zuurstofradicalen.

Het ontstaan van de zogenaamde 'zuurstofradicale ziekten van de neonatologie' is de belangrijkste uitkomst bij onderzoek naar oxidatieve stress bij premature pasgeborenen. Om oxidatieve stress te meten, hebben we markers van oxidatieve stress gemeten in de urine, wat gemakkelijk beschikbaar is zonder nadelige gevolgen voor de premature pasgeborenen. Daarnaast hebben we de synthese van glutathion, de belangrijkste intracellulaire antioxidant, gemeten. Deze werd bepaald in de rode bloedcellen. Aangezien onderzoek bij premature pasgeborenen wordt beperkt door kleine bloedvolumes, is het daarom belangrijk om de GSH synthese betrouwbaar te kunnen meten in kleine samples. **Hoofdstuk 2** beschrijft de ontwikkeling van een nieuwe methode, waarbij zowel GSH als diens voorloper glycine gelijktijdig worden gemeten door middel van Liquid Chromatography Isotope Ratio Mass Spectrometry (LC-IRMS), waardoor het benodigde bloedvolume kon worden gereduceerd.

DEEL 2: OXIDATIEVE STRESS BIJ GEBOORTE

Het eerste doel van dit proefschrift was het verminderen van de oxidatieve stress als gevolg van het toedienen van zuurstof tijdens de resuscitatie (opvang en stabilisatie) direct na de geboorte van premature pasgeborenen (**Hoofdstukken 3-5**). Vanwege onrijpheid van de longen, hebben premature pasgeborenen na de geboorte vaak beademing met extra zuurstof nodig om voldoende oxygenatie (SpO_2; zuurstofverzadiging in het bloed) te bereiken. Echter, zuurstof tijdens de resuscitatie na geboorte, zelfs wanneer het slechts enkele minuten wordt toegediend, kan langdurige schadelijke effecten hebben. Eerder onderzoek bij voldragen pasgeborenen heeft aangetoond dat het risico op overlijden wordt gereduceerd indien 21% zuurstof wordt gebruikt in plaats van 100% zuurstof. Ook werd er een trend tot afname van het risico op hersenschade geconstateerd na resuscitatie met 21% zuurstof in vergelijking met 100% zuurstof. Hoewel in recente richtlijnen wordt geadviseerd om te starten met lagere zuurstofconcentraties, is de optimale fractie van ingeademde zuurstof (FiO_2) bij prematuren pasgeborenen tijdens resuscitatie nog onbekend.

Initiële FiO_2 tijdens de resuscitatie van premature pasgeborenen

Eerdere studies naar de optimale FiO_2 tijdens de resuscitatie van premature pasgeborenen vergeleken het gebruik van lage FiO_2, dat wil zeggen 21% of 30% zuurstof, met een hele hoge FiO_2 zoals 90% of 100% zuurstof. In deze studies moest de FiO_2 in de groep startend met lage zuurstof vaak worden verhoogd om voldoende oxygenatie bereiken, terwijl de FiO_2 in de groep startend met hoge zuurstof vaak verlaagd werd. Resuscitatie van premature pasgeborenen met een initiële FiO_2 van 30% leidde wel tot een verlaging van de oxidatieve stress markers en een verminderd risico op bronchopulmonale dysplasie in vergelijking met een initiële FiO_2 van 90%. Om de veiligheid en effectiviteit van het gebruik van lage zuurstofconcentraties tijdens de resuscitatie van premature pasgeborenen te bepalen, was de volgende doelstelling om 30% zuurstof te vergelijken met een gemiddelde FiO_2.

Voor dit doel hebben we een dubbelblind, gerandomiseerd onderzoek verricht, waarin we een initiële FiO_2 van 30% vergeleken met 65% voor de resuscitatie van premature pasgeborenen (**Hoofdstukken 3 en 4**). In dit onderzoek hebben we 193 premature pasgeborenen geïncludeerd met een zwangerschapsduur onder de 32 weken. De premature pasgeborenen werden gerandomiseerd voor een initiële resuscitatie met 30% of 65% zuurstof, waarvoor de arts was geblindeerd. In deze studie hebben we aangetoond dat resuscitatie van premature pasgeborenen met 30% zuurstof net zo veilig is als resuscitatie met 65% zuurstof, maar het biedt geen voordelen met betrekking tot de overleving zonder bronchopulmonale dysplasie. De hoeveelheid zuurstof toegediend in de hoge zuurstofgroep was wel significant hoger tijdens de eerste vijf minuten. In onze studie resulteerde de hogere zuurstofbelasting daadwerkelijk in een hogere SpO_2 in het bloed, wat betekent dat het toegediende zuurstof ook daadwerkelijk in het bloed aankomt. Daarnaast beschikken premature pasgeborenen niet over

voldoende (pulmonale) antioxidanten om de plotselinge blootstelling aan zuurstof te overwinnen, waardoor ze gevoeliger zijn voor oxidatieve (long) schade. Bij elkaar genomen kunnen we concluderen dat resuscitatie van premature pasgeborenen met een zwangerschapsduur onder de 32 weken bij voorkeur wordt uitgevoerd met een initiële FiO_2 van 30%, in plaats van 65%. Zodra de zuurstofverzadiging stijgt, is individuele aanpassing van de FiO_2 noodzakelijk op basis van SpO_2.

Hoewel we geen verschillen hebben aangetoond in klinische korte termijn uitkomsten en oxidatieve stress, zal de lange termijn uitkomst van deze kinderen laten zien of er subtiele verschillen zijn als gevolg van blootstelling aan verschillende zuurstofpercentages die zich niet manifesteerden in de neonatale periode. Als onderdeel van de gerandomiseerde studie, zullen we de neurologische ontwikkeling onderzoeken op 2 jarige leeftijd (**Hoofdstuk 5**). Momenteel is deze follow-up nog gaande. Deblindering zal worden uitgevoerd nadat alle kinderen zijn getest op neurologische ontwikkeling op 2 jarige leeftijd.

FiO_2 aanpassingen tijdens de resuscitatie van premature pasgeborenen

Na de initiële fase van de resuscitatie van prematuren, moet de FiO_2 vervolgens worden aangepast op basis van de SpO_2. In 2010 introduceerde de richtlijn van de European Resuscitation Council (ERC) doelstellingen voor de SpO_2 gedurende de eerste 10 minuten na geboorte. Deze SpO_2 doelstellingen zijn gebaseerd op SpO_2 waarden van gezonde, meestal voldragen kinderen die geen ademhalingsondersteuning tijdens de resuscitatie nodig hadden. Het is de vraag of deze SpO_2 doelstellingen ideaal zijn voor premature pasgeborenen. Het is aangetoond dat premature pasgeborenen die geen ademhalingsondersteuning nodig hebben tijdens resuscitatie na de geboorte een tragere stijging van de SpO_2 hebben. Het kan worden bediscussieerd wat de juiste SpO_2 doelstellingen zijn voor premature pasgeborenen; die van gezonde voldragen kinderen of die van premature pasgeborenen die geen ademhalingsondersteuning nodig hebben. Vooralsnog is de huidige ERC richtlijn het best beschikbare bewijs en dienen deze SpO_2 doelstellingen te worden gebruikt om de FiO_2 toediening te titreren tijdens de resuscitatie.

Om de SpO_2 doelstellingen te bereiken en in stand te houden, wordt de FiO_2 handmatig aangepast. Deze handmatige controle van de SpO_2 is echter moeizaam, zoals blijkt uit het feit dat op de neonatale intensive care unit de SpO_2 50% van de tijd buiten het doelbereik ligt. Tijdens de resuscitatie maken de onrijpheid van de longen, de noodzaak van een stijging van de SpO_2 en de complexe procedures van de resuscitatie het reguleren van de SpO_2 nog moeilijker dan op de neonatale intensive care unit. De huidige richtlijnen geven geen advies over de wijze waarop en op welk moment de FiO_2 aangepast dient te worden en hoewel sommige strategieën zijn gepubliceerd, moet hun effectiviteit nog bewezen worden.

Zoals beschreven in **Hoofdstuk 6**, blijkt het volgen van de SpO_2 doelstellingen tijdens de resuscitatie van premature pasgeborenen in de huidige klinische praktijk moeilijk. In deze observationele studie werden grote afwijkingen van de SpO_2 doelstellingen waargenomen tijdens de resuscitatie van 78 premature pasgeborenen. Tijdens de eerste minuten van

resuscitatie waren de afwijkingen waarschijnlijk het gevolg van ontbrekende of onbetrouwbare SpO_2-meting in de onmiddellijke postnatale fase en moeilijkheden in ventilatie van de onrijpe longen. De huidige SpO_2 doelstellingen van de ERC zijn enkele waarden in plaats van een range en kunnen nooit precies worden gehaald. Het lijkt dus beter om een aanvaardbaar doelbereik te definiëren en deze te evalueren in de klinische praktijk.

Conclusies

De initiële FiO_2 voor de resuscitatie van premature pasgeborenen is tot op heden onderzocht in meerdere onderzoeken met verschillende initiële FiO_2, verschillende zwangerschapsduur van geïncludeerde kinderen en verschillende SpO_2 doelstellingen. Uit deze studies kan worden geconcludeerd dat resuscitatie moet worden gestart met lage zuurstofconcentraties. Onze studie toont aan dat een initiële FiO_2 van 30% voor premature pasgeborenen tijdens de foetale naar neonatale overgang net zo veilig is als 65% zuurstof en resulteert in verminderde blootstelling aan zuurstof tijdens de resuscitatie, maar 30% zuurstof biedt geen voordelen met betrekking tot kortdurende klinische uitkomst en de oxidatieve stress. Op dit moment wordt op de lange termijn neurologische follow-up gewacht.

Ons studieontwerp had een potentieel belangrijke beperking. Omdat toestemming van de ouders voor de geboorte gevraagd diende te worden, hebben we de premature pasgeborenen met een verwachte vroeggeboorte geselecteerd die dus ook allemaal prenatale corticosteroiden ter bevordering van de longrijping hebben ontvangen. De gevallen met onverwachte acute vroeggeboorte, waarbij ook geen tijd was voor deze prenatale corticosteroïden, hebben we niet geïncludeerd. Prenatale corticosteroïden bevorderen de longrijping en hebben een positieve invloed op de antioxidante afweer in premature pasgeborenen. De niet-geïncludeerde kinderen zouden dus mogelijk meer zuurstof nodig hebben vanwege de onrijpheid van de longen, maar zijn ook gevoeliger voor schadelijke gevolgen van blootstelling aan zuurstof. Toekomstige studies zouden gebruik moeten maken van een uitgestelde toestemming, waarbij toestemming achteraf kan worden gevraagd, om zo ook kinderen zonder prenatale corticosteroïden te kunnen includeren. Daarnaast kan het combineren van gegevens van alle voltooide en lopende studies in een meta-analyse subtiele effecten blootleggen, omdat grote aantallen inclusies nodig zijn voor minder voorkomende resultaten, zoals sterfte.

Na de initiële FiO_2, moeten latere aanpassingen worden gebaseerd op individuele SpO_2 metingen. Tot op heden zijn er geen gerandomiseerde studies verricht naar het juiste SpO_2 doelbereik tijdens de resuscitatie van premature pasgeborenen. Om verschillende regimes in SpO_2 doelen te vergelijken, is het belangrijk dat de SpO_2 voldoende gereguleerd kan worden, wat in de huidige klinische praktijk moeilijk is gebleken. Nieuwe technologische ontwikkelingen kunnen helpen bij 'de regulatie van SpO_2. Door het tonen van de actuele SpO_2 en het SpO_2 doel samen op een scherm, worden afwijkingen van het doel en de trend van de werkelijke SpO_2 beter geïnterpreteerd hetgeen kan bijdragen tot een betere beheersing van de SpO_2. Een automatische SpO_2-regelaar is reeds beschikbaar voor gebruik op de neonatale intensive

care unit ($CliO_2$, CareFusion, San Diego, Verenigde Staten) en het ontwikkelen van vergelijkbare technologie voor gebruik tijdens de resuscitatie kan nuttig zijn.

DEEL 3: VOEDING EN OXIDATIEVE STRESS

Een andere manier om de schadelijke effecten van oxidatieve stress tegen te gaan, is het verbeteren van de antioxidanten door optimalisatie van de voeding van premature pasgeborenen. De productie van eiwitten, zoals de synthese van glutathion, is afhankelijk van voldoende aanwezigheid en beschikbaarheid van aminozuren. Aminozuren zijn de 'bouwstenen' waaruit eiwitten opgebouwd worden. Bovendien is een energie belangrijk voor de eiwitsynthese, aangezien eiwitsynthese een proces is dat veel energie verbruikt. Energietekorten zijn echter nog steeds gebruikelijk bij premature pasgeborenen in de eerste levensweek. Het optimaliseren van de initiële voeding door het verhogen van de aminozuur toediening en het vroeg starten van toedienen van vetten zouden mogelijk weerstand tegen oxidatieve stress kunnen verbeteren door een verhoogde glutathion synthese.

Aminozuren

Frans te Braake et al. toonden eerder aan dat vroege toediening van 2.4 g/(kg•d) aminozuren aan premature pasgeborenen resulteerde in verhoogde concentratie van glutathion op de tweede levensdag, zonder een gelijktijdige stijging van de synthese van glutathion. In navolging van deze studie hebben we in **Hoofdstuk 7** laten zien dat de glutathion concentraties enkele uren na de geboorte significant lager zijn vergeleken met dag 2 in premature pasgeborenen die 2.4 g/(kg•d) aminozuren toegediend kregen vanaf geboorte. Deze stijging van de glutathion concentratie konden we niet verklaren door een verhoogde synthese van glutathion, aangezien deze op beide dagen niet verhoogd was. Dit suggereert dat de verhoogde glutathion concentratie na de initiële lage concentraties na de geboorte het gevolg kan zijn van een verminderd glutathion verbruik als gevolg van het toedienen van aminozuren. Mogelijke verklaringen hiervoor zijn het feit dat aminozuren, zoals methionine en cysteïne, op zichzelf kunnen dienen als antioxidant of dat de verhoogde beschikbaarheid van aminozuren de synthese van andere antioxidanten zoals albumine synthese stimuleert. Naast antioxidante eigenschappen, dient glutathion ook als een cysteïne reservoir. Het is mogelijk dat een toename van de beschikbaarheid van cysteïne door de verhoogde toediening van aminozuren, leidt tot een verminderde afbraak van glutathion om cysteïne vrij te genereren. Zoals aangetoond in **Hoofdstuk 8**, had het verder verhogen van aminozuur toediening tot 3.6 g/(kg•d) geen extra effect op glutathion beschikbaarheid of synthese.

Vetten

Vetten zijn een belangrijk onderdeel van de voeding van premature pasgeborenen, omdat zij de essentiële vetzuren leveren en een grote bron zijn van calorieën. Omdat vetten de belangrijkste bron van energie worden binnen enkele uren na toediening, kan vroege toediening van vetten resulteren in het sparen van eiwitten. In **Hoofdstuk 8** hebben we aangetoond dat het verhogen van de energie door middel van het toedienen van vetten direct vanaf de geboorte niet leidt tot een verhoogde glutathion synthese.

Vetten zijn gevoelig voor oxidatie en kunnen zo leiden tot verhoogde oxidatieve stress. De isofuranen and neurofuranen, markers van vetoxidatie, waren verhoogd op dag 2 in de groepen die vetten vanaf geboorte kregen toegediend. Deze verschillen verdwenen echter op de opvolgende dagen en de andere markers van oxidatieve stress waren niet verschillend tussen de groepen. Daarnaast resulteerde het vroeg starten van vetten niet in een verschillende klinische uitkomst. Samengevat lijkt het vroeg toedienen van vetten vanaf de geboorte geen relevant effect te hebben op oxidatieve stress in premature pasgeborenen.

Naast de timing van toediening van vetten, bestudeerden we ook het effect van het type vetemulsie dat werd toegediend. Traditionele vetemulsies worden hoofdzakelijk vervaardigd uit sojaolie en zijn zeer rijk aan ω-6 meervoudig onverzadigde vetzuren (PUFA's), die zeer gevoelig zijn voor vetoxidatie. Een multicomponent vetemulsie, bestaande uit sojaolie, middellange keten triglyceriden, olijfolie en visolie, met een hogere toegevoegde α-tocoferol (vitamine E) zou een positief effect kunnen hebben op oxidatieve stress. Echter, literatuur waarin het effect op oxidatieve stress van een multicomponent vetemulsie werd vergeleken met een pure sojaolie emulsie is tegenstrijdig. In de meeste van deze studies werden de vetten ofwel vanaf de geboorte in lage doses toegediend, of werden de vetten pas na een aantal dagen gestart. We voerden daarom dubbelblind gerandomiseerd onderzoek uit waarin we een multicomponent vetemulsie vergeleken met een pure sojaolie emulsie met een startdosering van 2 g/(kg•d) vanaf de geboorte (**Hoofdstuk 9**). Concentratie en synthese van glutathion waren niet verschillend na toediening van de twee vetemulsies vanaf de geboorte bij premature pasgeborenen. Behalve een verlaagde concentratie van de isofuranen, waren alle markers van oxidatieve stress gelijk tussen de twee verschillende vetemulsies. Daarnaast is in een recente meta-analyse aangetoond dat het type vetemulsie geen effect heeft op het ontstaan van neonatale ziekten. Alhoewel de consequenties van het verschil in isofuranen onbekend is, lijkt het toediening van een multicomponent vetemulsie niet te resulteren in een duidelijke vermindering van oxidatieve stress bij premature pasgeborenen.

Conclusies

Het starten van aminozuren vanaf de geboorte resulteerde in een verhoogde glutathion concentratie, waarschijnlijk als gevolg van een verminderde glutathion consumptie. Het is bekend dat de beschikbaarheid van extracellulaire aminozuren het verlies van glutathion door oxidatie onderdrukt. Derhalve limiteert de verhoogde beschikbaarheid van aminozuren het recyclen

van glutathion en dus de noodzaak voor synthese van glutathion. Er lijkt echter een limiet te zitten aan dit effect op glutathion, aangezien het toedienen van hoge doses aminozuren geen additief effect had.

Het verhogen van de energie door het vroeg starten van vetten resulteerde ook niet in een verhoogde glutathion synthese. Echter, de timing en het type van vetemulsie dat wordt toegediend aan premature pasgeborenen lijkt niet van aanzienlijke invloed op oxidatieve stress. Waarschijnlijk is oxidatieve stress al zo sterk verhoogd bij premature pasgeborenen dat de vroege toediening van vetten geen aantoonbaar effect hebben. Maar uit de lange termijn follow-up van de patiënten moet blijken of het kleine verschil in isofuranen en neurofuranen van klinische betekenis zijn.

We kunnen concluderen dat aanpassing van de voeding slechts een klein effect heeft op de synthese van glutathion bij premature pasgeborenen. Een maximale capaciteit van de synthese als gevolg van onrijpheid van de betrokken enzymen is niet de enige verklaring hiervoor, omdat premature pasgeborenen wel degelijk in staat lijken om glutathion te kunnen synthetiseren. Een tekort aan substraat zou moeten zijn ondervangen door een verhoogde toediening van aminozuren, maar mogelijk is de samenstelling van de aminozuren niet optimaal geweest. Eiwitsynthese is afhankelijk van de beschikbaarheid van alle benodigde aminozuren en de snelheid van de eiwitsynthese wordt dus bepaald door het eerste limiterende aminozuur.

Momenteel worden studies uitgevoerd om de behoefte van de individuele aminozuren bij premature pasgeborenen vast te stellen en toekomstige studies moeten het effect van verschillende aminozuur samenstellingen op basis van deze gegevens onderzoeken. Een andere aanpak van toekomstig onderzoek naar modulatie van oxidatieve stress via de voeding is de aanvulling van vitamines en andere antioxidanten bij premature pasgeborenen. Echter, tot op heden zijn de resultaten van dergelijk onderzoek niet eenduidig en is verder onderzoek dus belangrijk.

DEEL 4: ANDERE MODULATOREN VAN OXIDATIEVE STRESS

Het effect van neonatale infectie op oxidatieve stress

Een groot deel van premature pasgeborenen zal gedurende de eerste levensweken een infectie ontwikkelen, waarbij de gerapporteerde incidentie tussen 21 tot 36% ligt. Ondanks grote verbeteringen in de neonatale zorg, blijven infecties een belangrijke oorzaak van morbiditeit en overlijden bij premature pasgeborenen. Infecties bij premature pasgeborenen zijn geassocieerd met een nadelige neurologische ontwikkeling op 2-jarige leeftijd. De relatie tussen neonatale infectie en de nadelige ontwikkeling is waarschijnlijk multifactorieel, maar oxidatieve stress zou een belangrijke oorzaak kunnen zijn. Tijdens een infectie is de vorming van zuurstofradicalen verhoogd als onderdeel van normale afweer tegen ziekteverwekkers. Overstimulatie van de productie van zuurstofradicalen of onvoldoende antioxidanten zijn bij volwassenen

en kinderen geassocieerd met een verhoogde morbiditeit en overlijden. Door onrijpe en onvoldoende antioxidanten zijn premature pasgeborenen gevoeliger voor oxidatieve stress. Verhoogde vorming van zuurstofradicalen tijdens een infectie zou derhalve een belangrijke factor kunnen zijn in de nadelige lange-termijn uitkomst bij premature pasgeborenen na een neonatale infectie. In **Hoofdstuk 10** hebben we aangetoond dat infectie bij premature pasgeborenen niet resulteerde in een veranderde glutathion concentratie en synthese. Ook de markers van oxidatieve stress waren niet verschillend.

Hierbij valt op te merken dat het studie ontwerp wellicht niet optimaal was. De studie was opgezet om verschillen in glutathion synthese te detecteren en daarom waren niet voldoende kinderen geïncludeerd om betrouwbaar verschillen in oxidatieve stress op te sporen. Tegenwoordig zijn er minder invasieve methoden beschikbaar om markers van oxidatieve stress te meten, zoals bijvoorbeeld oxidatieve stress markers in de urine. Hierdoor zouden toekomstige studies meer premature pasgeborenen kunnen includeren en is het ook mogelijk om 'gezonde' premature pasgeborenen als controle groep te gebruiken. Onze resultaten geven geen verklaring voor de relatie tussen een neonatale infectie en een nadelig lange-termijn uitkomst. Het is van groot belang om andere mechanismen verder te verkennen, zoals de productie van pro-inflammatoire cytokines, waarvan bekend is dat ze neurotoxisch zijn.

Determinanten van glutathion synthese

Een kortere zwangerschapsduur, een lager geboortegewicht en mannelijk geslacht worden geassocieerd met een slechtere uitkomst. Dit effect zou deels gemedieerd kunnen zijn door verschillen in de antioxidanten capaciteit. Omdat oxidatieve stress wordt geassocieerd met zowel morbiditeit en overlijden bij premature pasgeborenen, veronderstelden we dat geslacht, zwangerschapsduur en geboortegewicht van invloed zijn op metabolisme van glutathion. Echter, de verschillen in uitkomsten gerelateerd aan geslacht, zwangerschapsduur en geboortegewicht lijken niet te worden verklaard door verschillen in glutathion synthese (**Hoofdstuk 11**).

Een interessante bevinding was het feit dat de GSH-GSSG verhouding (verhouding tussen glutathion en zijn geoxideerde vorm) lager was bij meisjes vergeleken met jongens, wat in tegenstelling is tot eerder gerapporteerde verschillen van oxidatieve stress in relatie tot geslacht. Dit dient dan ook verder te worden uitgezocht.

Het proces van zuurstof toediening

Een andere belangrijke factor die leidt tot een verhoogde oxidatieve stress bij premature zuigelingen, is de toediening van zuurstof tijdens hun verblijf op de neonatale intensive care unit. Door de onrijpe longen hebben premature pasgeborenen vaak extra zuurstof nodig om een voldoende oxygenatie van het bloed te bereiken en te behouden. Helaas heeft aanvullende zuurstoftherapie een zeer klein therapeutisch bereik, aangezien zowel te veel als te weinig zuurstof ernstige gevolgen kunnen hebben voor de uitkomst van premature pasgeborenen.

Momenteel wordt de toegediende zuurstof handmatig aangepast om de SpO_2 binnen het doelbereik te houden. Het proces van handmatige bediening van de zuurstof kan echter aan fouten onderhevig zijn. Dit wordt onderstreept door het Neonatology System for Analysis and Feedback on medical Events (NEOSAFE), waar (bijna-) ongevallen door personeel op anonieme en vrijwillige basis worden gerapporteerd. In 2010 had slechts 6% van de gerapporteerde ongevallen betrekking op 'extra zuurstof therapie'. Echter, deze rapporten waren wel verantwoordelijk voor 90% van de (bijna-) ongevallen die werden gecategoriseerd als 'meest riskant'. Daarom hebben we een zogenaamd Failure Mode and Effects Analysis (FMEA) uitgevoerd om gevaren in het proces van extra zuurstoftherapie bij premature pasgeborenen opgenomen op de neonatale intensive care unit te evalueren en om aanbevelingen voor verbetering te geven (**Hoofdstuk 12**).

De top tien risico's kunnen allen worden gecategoriseerd binnen drie hoofdthema's: onjuiste aanpassing van de zuurstof, onjuiste alarmgrenzen voor SpO_2 op de neonatale intensive care unit en onjuiste alarmgrenzen voor SpO_2 op patiëntmonitoren voor tijdelijk gebruik. De FMEA resulteerde in aanbevelingen voor zowel scholing als technische verbeteringen. Deze omvatten suggesties voor (veranderingen in) protocollen op alarmgrenzen en handmatige FiO_2 aanpassingen, het opleiden van medewerkers in de gevaren van extra zuurstof en technische verbeteringen in beademingsapparaten en patiëntmonitoren.

CONCLUSIE

Verhoogde oxidatieve stress kan nadelige gevolgen hebben, met name bij premature pasgeboren die postnataal de normale intra-uteriene ontwikkeling moeten doormaken in een zuurstofrijke omgeving. Oxidatieve stress heeft echter ook een belangrijke functie in normale processen, zoals bij cardiopulmonale adaptatie bij de geboorte, de verdediging tegen ziekteverwekkers en bij inter- en intracellulaire signalering. Er lijkt dus een delicaat evenwicht te bestaan tussen pathologische en fysiologische oxidatieve stress. Het is duidelijk dat oxidatieve stress een belangrijke rol speelt in neonatale morbiditeit en overlijden. De mechanismen van oxidatieve stress en de antioxidanten afweer zijn complex en verder onderzoek blijft noodzakelijk.

Dankwoord

Het duurde even, maar het is af! Het includeren van 200 prematuren bleek niet zo eenvoudig, maar het resultaat is er naar. Dit was niet mogelijk geweest zonder de hulp en steun van velen.

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Mijn onderzoek was niet mogelijk geweest zonder alle neonatologen, arts-assistenten en verpleegkundig specialisten van de afdeling neonatologie. Ga er maar aan staan, in een acute situatie geblindeerd zuurstof geven aan, soms extreme, prematuren. Dank jullie wel voor al die keren dat jullie mijn 'knop' hebben gebruikt. Natuurlijk wil ik ook alle verpleegkundigen van de afdeling neonatologie bedanken voor het verzamelen van alle urine en al die keren dat ze voor mij bloed wilden afnemen. Tijdens mijn onderzoek heb ik veel tijd doorgebracht op 'de zwangere' om toestemming te vragen voor mijn onderzoek en ik wil dan ook de artsen en verpleging bedanken voor hun gastvrijheid. Ik denk dat dit een mooi voorbeeld is van samenwerking binnen het Moeder- en Kind centrum.

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Denise

Curriculum Vitae

Denise Rook was born on May 8, 1979 in Willemstad, Curacao. After moving to the Netherlands at the age of six, she grew up in Pijnacker and completed grammar school in 1997 at the Zandvliet College in The Hague. Before studying medicine, she obtained her MSc degree in Biomedical Sciences at the University of Leiden in 2001. From 2001 to 2005, she went on to study Medicine at the University of Leiden.

After completing medical school, she started working as a resident pediatrics at the Westeinde Hospital in The Hague and at the Reinier de Graaf Hospital in Delft. Her love for biomedical research and Neonatology culminated in a PhD research project, entitled 'Oxidative stress in preterm infants', at het neonatal intensive care unit at the Erasmus MC - Sophia's Childrens Hospital in Rotterdam, the Netherlands (supervisors Prof. Dr. J.B. van Goudoever and Dr. M.J. Vermeulen). Since august 2012, she is working as resident pediatrics (ANIOS) at the Erasmus MC - Sophia's Childrens Hospital. In January 2013, she started her pediatric residency in training (AIOS) at the Erasmus MC - Sophia's Childrens Hospital.

List of Publications

Rook D, Vlaardingerbroek H, Muizer Y, Dorst K, Kuligowski J, Escobar J, Vento M, Vermeulen MJ, van Goudoever JB, Schierbeek H. A multicomponent lipid emulsion containing a mixture of soybean oil, medium-chain triglycerides, olive oil and fish oil does not reduce oxidative stress in very low birth weight infants; a double-blind randomized controlled trial. *Submitted.*

Rook D, Vlaardingerbroek H, Muizer Y, Dorst K, Kuligowski J, Escobar J, Vento M, van Goudoever JB, Schierbeek H. Increased amino acid and early lipid administration do not up-regulate glutathione synthesis, nor increase oxidative stress in very low birth weight infants. *Submitted.*

Vlaardingerbroek H, Roelants JA, **Rook D**, Dorst K, Schierbeek H, Vermes A, Vermeulen MJ, van Goudoever JB, van den Akker CHP. Adaptive regulation of amino acid metabolism in very low birth weight infants. *Submitted*.

Vlaardingerbroek H, Schierbeek H, **Rook D**, Vermeulen MJ, Dorst K, Vermes A, Van Goudoever JB, Van den Akker CHP. Albumin synthesis is enhanced by early parenteral lipid and high dose amino acid administration to very low birth weight infants. *Submitted.*

Rook D, De Groot J, Schierbeek H, Longini M, Buonocore G, van Goudoever JB. Glutathione kinetics and oxidative stress markers in preterm infants with nosocomial sepsis. *Submitted.*

Kuligowski J, Torres-Cuevas I, Quintás G, **Rook D**, van Goudoever JB, Cubells E, Asensi M, Vento M, Escobar J, Validation of a rapid and non-invasive method for the assessment of oxidative damage to proteins and DNA in urine of newborn infants using UPLC-MS/MS. *Submitted.*

Van der Eijk AC, **Rook D**, Schutte S, Dankelman J, Simonsz HJ, Smit BJ. Pulse oximetry alarm limits in extremely low birth weight infants; When do deviations from the protocol occur? *Submitted.*

Rook D, Schierbeek H, Vento M, Vlaardingerbroek H, van der Eijk AC, Longini M, Buonocore G, van Goudoever JB, Vermeulen MJ. No differences in clinical outcome or oxidative stress between an initial fraction of inspired oxygen of 30% and 65% during resuscitation of preterm infants after birth: a double-blind, randomized controlled trial. *Submitted.*

Vlaardingerbroek H, Vermeulen MJ, **Rook D**, van den Akker CHP, Dorst K, Wattimena JL, Vermes A, Schierbeek H, van Goudoever JB. Safety and efficacy of early parenteral lipid and high dose amino acid administration to very low birth weight infants, a randomized controlled trial. *Submitted.*

Rook D, van Goudoever JB. Oxidative stress and Glutathione synthesis rates in early postnatal life. In: Studies on Perinatal & Prenatal Disorders. *In press.*

Goos TG, **Rook D**, van der Eijk AC, Kroon AA, Pichler G, Urlesberger B, Dankelman J. Reiss IKM. Resuscitation of preterm infants: Are we able to follow the oxygen saturation guidelines? *In press.*

Van der Eijk AC, **Rook D**, Dankelman J, Smit BJ. Defining hazards of supplemental oxygen therapy in neonatology using the FMEA tool. *In press.*

Rook D, Schierbeek H, van der Eijk AC, Longini M, Buonocore G, Vento M, van Goudoever JB, Vermeulen MJ. Resuscitation of very preterm infants with 30% vs. 65% oxygen at birth: study protocol for a randomized controlled trial. *Trials 2012, 13(1):65.*

Rook D, Te Braake FWJ, Schierbeek H, Longini M, Buonocore G, van Goudoever JB. Glutathione Synthesis Rates in the Immediately Postnatal Phase. *Pediatr Res 2010; 67(4): 407-11.*

Schierbeek H, **Rook D**, Te Braake FWJ, Dorst KY, Voortman G, Godin JP, Fay LB, van Goudoever JB. Simultaneous analysis of 13C-glutathione as its dimeric form GSSG and its precursor [1- 13C] glycine, in neonates using Liquid Chromatography Coupled to Isotope Ratio Mass Spectrometry (LC-IRMS*). Rapid Commun Mass Spectrom 2009, 23(18): 2897-902.*

Rook D, Van der Lely N. Alcoholintoxicatie bij jongeren: een groeiend probleem. *Psychiatrie centraal, zomereditie 2007*

Van Dunné FM, Lard LR, **Rook D**, Helmerhorst FM, Huizinga TW. Miscarriage but not fecundity is associated with progression of joint destruction in rheumatoid arthritis. *Ann Rheum Dis 2004; 63(8): 956-60.*

Aarbiou J, Ertmann M, van Wetering S, van Noort P, **Rook D**, Rabe KF, Litvinov SV, Krieken JHJM, de Boer WI, and Hiemstra PS. Human neutrophil defensins induce lung epithelial cell proliferation in vitro. *J Leukoc Biol 2002; 72(1): 167-74.*

PhD Portfolio

Name	Denise Rook
Erasmus MC department	Pediatrics - Neonatology
PhD period	August 2007 – August 2012
Promotor	Prof. Dr. J.B. van Goudoever
Copromotor	Dr. M.J. Vermeulen

	Year	Workload (ECTS)
PhD training		
Courses		
Good Clinical Practice, Rotterdam, the Netherlands	2008	0.8
Biomedical Research Techniques, Rotterdam, the Netherlands	2008	0.1
Classical Methods for Data-analysis, Rotterdam, the Netherlands	2009	5.7
Consultatiecentrum Patiëntgebonden Onderzoek (CPO) course, Rotterdam, the Netherlands	2010	0.3
Intensive Course In Tracer Methodology , Stockholm, Sweden	2010	0.9
Biomedical English Writing and Communication, Rotterdam, the Netherlands	2011	4
Presentations		
Poster presentation, PAS conference, Honolulu, Hawaii, USA	2008	0.9
Poster - symposium, ESPR conference, Nice	2008	0.9
Oral presentation, Basis meeting, Brugge, Belgium	2009	0.6
NVK Young Investigators Meeting, Veldhoven, The Netherlands	2009	0.3
Oral presentation, DSPM, Utrecht, The Netherlands	2009	0.6
Oral presentation, Grand Round, Siena, Italy	2010	0.3
Oral presentation, EAPS conference, Young Investigator Award	2010	0.9
Oral presentation, Neonatal Fellow Meeting, Groningen, The Netherlands	2011	0.6
Oral presentation, PAS conference, Denver, USA	2011	0.9
Poster - symposium, ESPGHAN conference, Sorrento, Italy	2011	0.6
Poster - symposium, EAPS conference, Istanbul, Turkey	2012	0.9
International conferences		
Pediatric Academic Societies' (PAS) Annual Meeting, Honolulu, Hawaii, USA	2008	1.4
2nd Congress of the European Academy of Paediatrics (EAP), Nice, France	2008	1.1
50th Annual Meeting of the European Society for Paediatric Research (ESPR), Hamburg, Germany	2009	1.4

3rd Congress of the European Academy of Paediatric Societies (EAPS), Copenhagen, Denmark	2010	1.4
Pediatric Academic Societies' (PAS) Annual Meeting, Denver, USA	2011	1.1
44th Annual Meeting of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), Sorrento, Italy	2011	1.1
4th Congress of the European Academy of Paediatric Societies (EAPS), Istanbul, Turkey	2012	1.1

Seminars and workshops

Erasmus MC PhD Day	2008	0.1
Benelux Association of Stable Isotope Scientists (BASIS) Meeting, Arnhem, the Netherlands	2008	0.6
Sophia Research Day, Rotterdam, the Netherlands	2008	0.1
Benelux Association of Stable Isotope Scientists Meeting (BASIS), Brugge, Belgium	2009	0.6
Dutch Society Perinatal Medicine (DSPM), Utrecht, the Netherlands	2009	0.3
Sophia Research Day, Rotterdam, the Netherlands	2009	0.1
NVK Young Investigators Meeting, Veldhoven, the Netherlands	2009	0.3
Symposium Extreme Prematuur, Rotterdam, the Netherlands	2010	0.3
2e Dutch Neonatal Fellow Meeting, Groningen, the Netherlands	2011	0.6
2nd Brussels Neonatology Symposium, Brussels, Belgium	2011	0.3
New Insights into Neonatal Resuscitation, Leiden, the Netherlands	2012	0.3

Other

Board Sophia Onderzoekers Vertegenwoordiging (SOV)	2009	1.4
HFMEA analyse	2011	1.4

Teaching activities

Lecturing

Clinical lectures for nurses and medical staff, Department of Pediatrics and Department of Obstetrics, Rotterdam, the Netherlands	2008-2010	0.3
Presentation on Nurses IC Symposium	2011	0.3

Supervising Master's theses

Medical student J. de Groot	2009	1.4
Medical student J. Roelants	2010	1.4
Student Biomechanical engineering T. Goos	2011	1.4
Medical student Y. Muizer	2011	1.4