The Need To Feed

Effects of amino acid administration on protein metabolism and antioxidant defense in preterm infants

Franciscus Wilhelmus Johannes te Braake

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Effects of amino acid administration on protein metabolism and antioxidant defense in preterm infants

De noodzaak tot voeden

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	Prof.dr. H.J.G. Boehm
	Prof.dr. D. Tibboel

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Frans WJ te Braake, Chris HP van den Akker, Maaike A Riedijk, and Johannes B van Goudoever

The first two authors contributed equally
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Prematurity

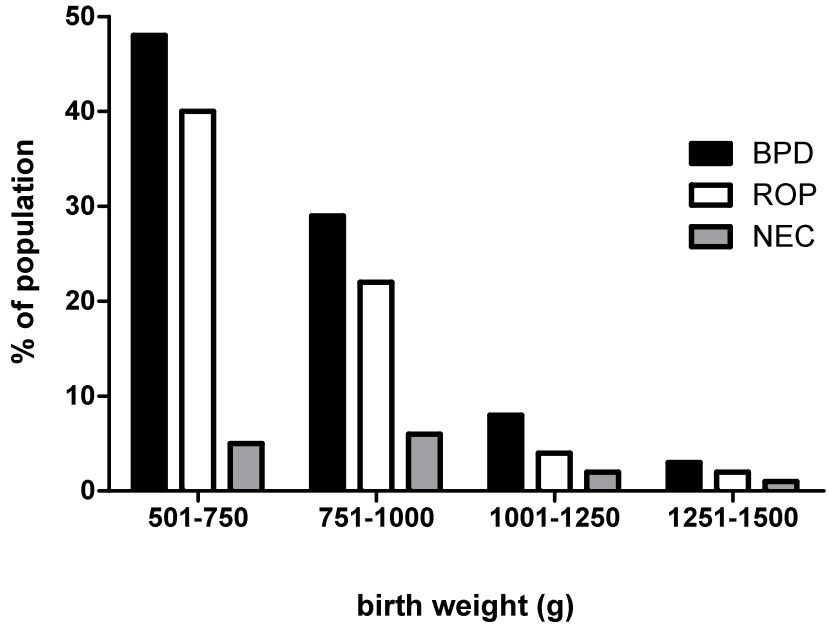
Neonates with a gestational age <37 weeks are called preterm. This thesis, however, presents studies in *very* preterm infants (gestational age <28 weeks), some being even *extremely* preterm (gestational age <26 weeks). Apart from gestational age, preterm neonates can be classified according to birth weight: low birth weight infants, weighing <2500 gram; very low birth weight infants, weighing <1500 gram; and extremely low birth weight infants, weighing <1000 gram. The cause of prematurity is multifactorial. Maternal factors (preeclampsia, life style, age, infection), pregnancy factors (placenta previa, premature rupture of membranes, polyhydramnion) , as well as fetal factors (multiple gestation, congenital anomalies) play a role (1).

Our ability to treat increasingly preterm infants has improved significantly, as reflected by the substantial increase in survival over the years. In two cohorts of VLBW infants born in 1989 and 1994, it was reported that mortality decreased nearly 50% for VLBW infants in 5 years (2). One third of this decline was attributed to improved condition on admission, reflecting improved obstetric and delivery room care. The other two thirds of the decline in mortality was attributed to intensive care treatment, including improved respiratory and cardiovascular support, such as surfactant use, application of antenatal steroids and availability of ultrasound techniques.

Increased survival, on the other hand, poses new challenges to neonatal care. As more preterm infants survive the direct neonatal period, prematurity related morbidity increases (3). Figure 1 shows incidence of a number of major prematurity related diseases in our neonatal intensive care unit. Apart from a certain maladjustment to neonatal life arising from immature organ systems, the etiology of many of these diseases, such as bronchopulmonary dysplasia, periventricular leukomalacia, and retinopathy of prematurity is largely unknown.

Compared to other innovations in neonatology, such as artificial ventilation, progress in the field of nutrition has been lagging behind, probably since malnutrition or even complete absence of nutrients has no short-term life threatening consequences. The priority for adequate nutrition as soon as possible after birth, in particular for protein accretion, is increasingly being recognized. However, this is complicated by the relative intolerance preterm infants exhibit to enteral nutrition. For this reason, they are primarily dependent on parenteral nutrition in the immediate postnatal phase.

Figure 1. Incidence of bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP) and necrotizing enterocolitis (NEC) divided by birth weight in a cohort of 670 infants, born in the Sophia Children's Hospital between 2002 and 2006.



Parenteral amino acid solutions for the preterm infant

From past to present

Parenteral nutrition became available for routine use in the neonatal intensive care units (NICUs) in the early 1970s, and development is still continuing. The first amino acid (AA) solutions were found to cause metabolic disturbances in newborn infants (4). Studies reporting these adverse effects had and still have a profound effect on nutritional policies. Though it was recognized that withholding proteins resulted in a catabolic state, AAs were being withheld assuming that the preterm infant was 'intolerant' to AA solutions. We have now come to realize that both manufacturing mode and composition of the AA solution are likely to have caused complications such as hyperammonemia and metabolic acidosis, rather than the AAs per se. In fact, after the umbilical cord is cut following preterm birth, the concentrations of essential AAs start to fall rapidly. Nowadays, we know this can trigger a response referred to as 'metabolic shock', a starvation response of the body which is accompanied by irrepressible endogenous glucose production, which causes glucose intolerance and further consumption of AAs

for gluconeogenic substrates. Indeed, the need for AAs may never be more urgent that directly following birth.

Nevertheless, fear of metabolic derangements is still firmly rooted in clinical practice.

Guidelines, such as presented by the Committee on Nutrition of the American Academy of Pediatrics, have stressed the importance of AA administration to preterm neonates already since the 1970s (5). The goal stated at that time remains valid today: a postnatal growth rate that duplicates fetal growth rate. An additional present aim is mimicking body composition of the age-matched fetus. However, the exact timing of initiation of AA supplementation was not addressed until recently. Recent guidelines state that even one day of starvation can be detrimental to the preterm infant (6).

Intrauterine nutrition

Through the umbilical cord, the fetus receives a continuous supply of AAs. While preterm birth causes this supply to cease abruptly, in the age-matched fetus the ongoing delivery of AAs is vital for growth and neurodevelopment. Yet, preterm infants often do not receive sufficient amounts of AAs, or even do not receive them at all during the first postnatal days. Clinical issues such as patent ductus arteriosus and respiratory distress with a subsequent strict fluid management might complicate adequate nutrient provision.

Nutritional uptake in utero is large, not only for accretion of new tissue and a high oxidation rate, but also for replacement of body water with protein and fat. Water content of fetal tissue will drop from 89% at 24 wks to 74% at 40 wks gestation. This drop is counterbalanced by a rise in lipid content from 0 to 11 % in the last trimester, and a rise in protein content from 8.8% to 12% (7).

AA contribution to human fetal oxidation is largely unknown and the few available data are contradictory. Obligatory nitrogen excretion in fasting premature neonates is approximately 0.6 - 1.0 g/(kg·d) (8, 9). Animal fetal research in physiological conditions indeed demonstrates that intrauterine AA oxidation is much higher and that uptake is far beyond AA requirements for body accretion (10, 11). The human fetus also showed considerable intrauterine urea production (12). Total AA uptake is 3.5 to 4.0 g AA/(kg·d). Protein requirements at two months of age after term birth only slightly decrease to approximately 2.5 to 3.0 g/(kg·d) as tissue protein content does not further increase.

It must be noted that these values are derived from tissue composition of deceased fetuses or newborns whose growth might also have been affected; thus these figures might be underestimations. Nevertheless, we may still assume that the fetus receives a diet rich in protein and poor in fat. Yet, present postnatal nutritional strategies dictate

the preterm infant be given a high fat, high carbohydrate, and moderately high protein diet. While a high caloric diet indeed stimulates a preterm infant's growth, mass accretion would have been different in composition had the infant still been in utero. Indeed, preterm infants were found to gain larger than desirable fat deposition after birth (13, 14).

Achieving a body composition closer resembling fetal body composition, usually implies a larger AA intake. This should be accomplished as soon as possible after birth. Note, however, that growth failure is not the same as weight loss. The initial weight loss in the first postnatal days represents rearrangements of body fluids necessary for adapting to extrauterine life, rather than catabolism.

Early amino acid administration

In early studies AAs were initiated not until after one week in the smallest infants (15) or after 3 days in 1700 g-infants (16), leaving infants dependent on exogenous glucose only for their metabolism during the bridging period. With the introduction of solutions specifically designed for neonates (17), researchers started to study effects of shortening time span of withholding AAs (8, 18-21). In two separate studies, Van Goudoever et al. and Murdock et al. were the first to administer AAs immediately after birth, though using only 1.15 and 1.35 g/(kg·d) in infants weighing a mean 1400 g and 1500 g, respectively (9, 22). Neither of these or the other studies reported metabolic acidosis, hyperaminoacidemia, or, when measured, hyperammonemia. Beneficial effects – improvements in nitrogen balance, stable isotope balance, or plasma AA profile – were observed in all studies.

NICUs now usually start AA infusion in premature infants between 0 and 36 hours after birth. However, amounts at which AA administration are initiated may vary considerably and not seldom are started at only 0.5 or 1.0 g/(kg·d) increasing step wisely up to 3.0 g/(k·d) over several days. The motivation for the stepwise increase of AA intake is not empirically based, but rather dictated by fluid limitations, concerns of intolerance, and fear of hyperglycemia in case of mixed glucose/AA solutions.

However, over the years quality of intravenous AA solutions has improved, and so has the general condition of the preterm infant before and immediately after delivery. Yet there is a need for more research on nutrition in the immediate postnatal phase. Indeed, the sudden change from a usually well-fed intrauterine state to the extrauterine environment, makes the sick premature most vulnerable and, therefore, in urgent need of balanced nutrition.

Conditionally essential amino acid requirements in neonates

AAs can be categorized into essential, non-essential and conditionally essential AAs.

An important factor in premature infant feeding is the biochemical immaturity. Several metabolic processes are not fully developed in utero and are activated after birth. The following AAs are therefore considered to be conditionally essential in preterm infants: arginine, cysteine, glutamine, glycine, proline, taurine and tyrosine. In this thesis, cysteine, glycine and taurine are of particular interest, since cysteine and glycine are substrates for glutathione synthesis, which will be discussed later, and taurine is a product of cysteine catabolism. These AAs will be shortly discussed below.

Cysteine

Cysteine is synthesized de novo from the essential AA methionine by the transsulfuration pathway. Cysteine is a sulfur-containing AA and has several metabolic functions: it is a precursor for taurine and, more importantly, for glutathione. Cysteine synthesis is assumed to be impaired in preterm infants due to low or absent activity of the enzyme cystathionase (23, 24). However, cystathionase is rapidly activated in the first month after birth (24, 25). Viña et al. reported that plasma cysteine levels were significantly lower in preterm infants receiving a cysteine-free PN solution intravenously compared to term infants (26). Stegink and Den Besten suggested that the gastro-intestinal tissues are an important site for cysteine synthesis (27). They showed that plasma cystine concentration dropped significantly in human adults fed a cystine-free diet intravenously and increased rapidly to almost normal during intragastric infusion. Cysteine requirement might be even higher in preterm neonates fed PN than term babies during the first days of life due to these two factors. Nevertheless, recent studies have shown that the transsulfuration pathway in VLBW neonates is active 48 hours after birth, whereas the capacity was directly related to neonatal maturity (28, 29). These studies suggest that cysteine is not a conditionally essential AA, but can be synthesized de novo by preterm infants. However, these results have to be confirmed in larger studies.

Cysteine is not stable in solution and oxidizes easily to cystine, which is insoluble, and most standard parenteral solutions therefore contain little cysteine or are cysteine-free, which puts parenterally fed infants at risk for cysteine deficiency.

To compensate for these low cysteine concentrations, parenteral solutions contain relatively high levels of methionine. Neonates receiving a high dose of methionine show high plasma methionine and low plasma cysteine concentrations. This implies that excess of methionine is not used for cysteine synthesis. Moreover, high methionine levels in rats induce functional and structural hepatic injury and could be an important factor in the pathogenesis in TPN-associated cholestasis (30).

N-acetyl-L-cysteine (NAC) is stable in solution but when it was supplemented to parenteral solutions, high concentrations of NAC were detected in the urine, confirming its low bioavailability (31). So, supplementation of NAC does not seem to be an adequate approach to increase the bioavailability of cysteine.

Glycine

Glycine is formed by reversible conversion from serine, which is synthesized de novo. It is extensively metabolized in the liver where it serves as an ammonia donor. It functions as an inhibitory neurotransmitter in the central nervous system and is also, like cysteine, a precursor for glutathione synthesis. The demand for glycine in preterm infants might be increased during critical illness or during oxidative stress. Glycine requirement then may be temporarily higher in these infants and might need to be supplemented in PN solution. Another indication for additional glycine supplementation is derived from [^{15}N] glycine studies in which, particularly in SGA infants, hardly any added supplemented tracer could be found in urinary urea (8, 32).

Taurine

Taurine is a small β-AA and is endogenously formed from cysteine. It is important for fetal neurological development but is not produced by the fetus. Taurine is not used for protein synthesis, but remains free in the intracellular water. Plasma values drop in infants receiving a taurine free PN solution. Taurine has several important functions and deficiency results in impaired fat absorption, bile acid secretion, retinal function, and hepatic function, all of which can be reversed by taurine supplementation (33). Cysteine sulfinic acid decarboxylase is the rate-limiting enzyme for taurine synthesis and its activity might be lower in preterm infants compared to adults. Also, as PN contains no or little cysteine, exogenous cysteine supplementation is not optimal for taurine production and, therefore, it is indeed conditionally essential.

Oxidative stress, antioxidants and related morbidity

Oxidants & Antioxidants – general aspects

The intrauterine environment is hypoxic relative to the extrauterine environment. More specific, the uterus has a low oxygen tension (pO_2 = 20-25 mm Hg) as compared to room air (pO_2 = 150-160 mm Hg) (34). Fetal and adult gene expression is different, enabling the fetus to thrive in this hypoxic intrauterine environment, while it is capable

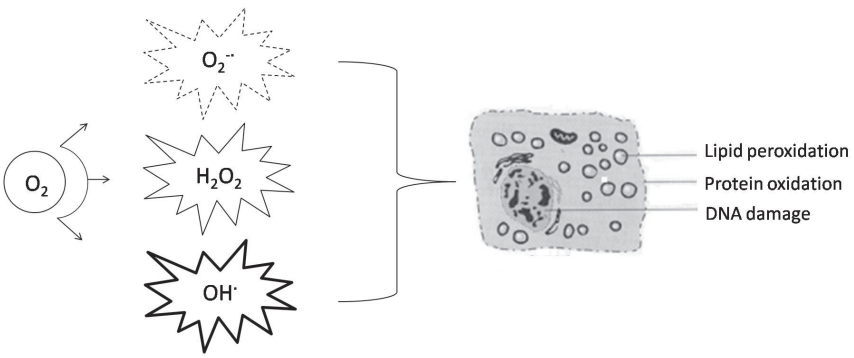
to anticipate to the relative hyperoxia following term birth. The latter primarily involves protection which is required against an increased load of oxidants evolving as by-products of oxidative metabolism, i.e. the reduction of O_2 to H_2O which takes place during the process of energy generation. These oxidants are referred to as *reactive oxygen species* (ROS), and include free radicals, such as superoxide ($O_2^{\cdot-}$) and the extremely reactive hydroxyl radical ($OH^{\cdot}$) as well as non-radical substances, such as hydrogen peroxide (H_2O_2). These ROS can extract electrons from other molecules rendering them either irreversibly damaged or new radicals which may continue the cascade. From this it can be concluded that oxygen, although required to sustain life, has toxic properties. Hence the phrase *oxygen paradox*.

It is important to note that, in the physiological state, ROS are present and maintained at low concentrations at which they are of benefit in regulating gene expression and several types of cellular signaling (35). In vitro, H_2O_2 and $O_2^{\cdot-}$ in extremely low concentrations stimulate growth of various cell types (36, 37). During fetal life, they lead to the digit individualization in developing limbs by means of carefully programmed apoptosis (38). In addition, neutrophils and macrophages contain high concentrations of free radicals which are released during inflammation in order to eliminate pathogens.

At higher concentrations, ROS inflict damage to cellular constituents, ultimately resulting in apoptosis (Figure 2). This damage can be quantified by measuring concentrations of specific markers, which in the healthy state are either absent or present in minimal amounts. These markers can reflect protein oxidation (e.g. advanced oxidized protein products, dityrosine), lipid peroxidation (e.g. malondialdehyde, isoprostanes), and DNA damage (e.g. 8-hydroxy-2'-deoxyguanosine).

Tissue damage evoked by ROS can be prevented by *reducing agents* or *antioxidants*. An antioxidant, by definition, can be "any substance that delays, prevents or removes oxidative damage to a target molecule" (39). Antioxidants can be classified according to many criteria: some can be synthesized *in vivo* whereas others should be consumed

Figure 2. ROS mediated cellular damage.



within the diet; they can act primarily in the intracellular or in the extracellular environment; they can be enzymatic or non-enzymatic.

Imbalance between oxidants and antioxidants in favor of the former is referred to as *oxidative stress*. In other words, oxidative stress is a physiological stress on the body that is caused by the cumulative damage done by ROS inadequately neutralized by antioxidants, which can either result from increased oxidant production, inadequate antioxidant production, or a combination of both.

A number of important antioxidants will be briefly discussed below:

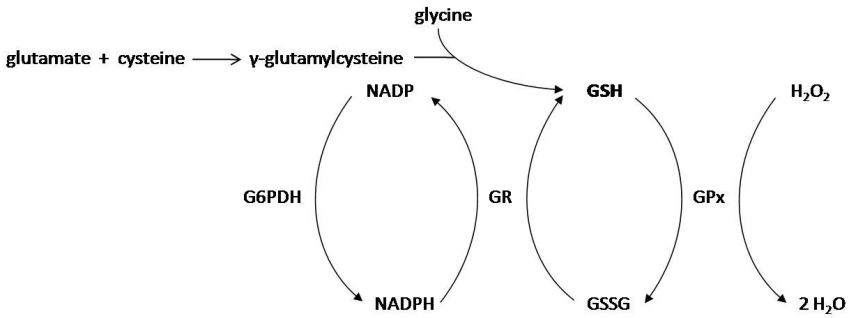
Glutathione

Glutathione (GSH) is a tripeptide composed of glutamate, cysteine, and glycine, by the consecutive action of the enzymes glutamate cysteine ligase (GCL) (EC 6.3.2.2) and glutathione synthase (EC 6.3.2.3). It is primarily an intracellular antioxidant, synthesized by virtually all mammalian cells. Cellular concentrations are extremely high, typically in the millimolar range. Its half life ranges from minutes to several days and is tissue specific (40-43). It increases during pathologic conditions such as oxidative stress. GSH can function as an antioxidant independently, by means of the cysteine moiety, which has a reducing sulfhydryl group. It scavenges ROS and can be considered as first line defense (44). In addition, GSH is a cofactor for numerous antioxidant enzymes, such as GSH-peroxidases (EC 1.11.1.9) and GSH-S-transferases (EC 2.5.1.18) which collectively form a second line defense system eliminating noxious byproducts of ROS. The monomeric form is the reduced, or active form of GSH. While reducing harmful ROS, GSH itself is oxidized to form the dimeric form, typically abbreviated as GSSG since the two GSH monomers are coupled by a disulfide bond. Intracellular GSSG concentrations are extremely low and the GSH/GSSH equilibrium is tightly controlled as it regulates a number of metabolic processes, such as enzyme activity and gene expression (45).

To maintain the reduced intracellular environment, GSSG will be either exported extracellularly, or recycled to GSH by GSH reductase (EC 1.8.1.7). This reaction requires NADPH as a co-factor, which is yielded from the pentose phosphate pathway. This whole process of reduction and oxidation is called the GSH redox cycle (Figure 3).

Although all cells synthesize GSH, the liver is the main producer and exporter, its export accounting for over 90% of total GSH turnover (46). GSH cannot be transported as a tripeptide across cellular membranes. For this reason, it seems of little benefit to supply GSH as part of parenteral nutrition to increase intracellular GSH concentrations. On the other hand, indirect transport and subsequent intracellular resynthesis is facilitated by the γ-glutamyl cycle in a few organs such as the kidney and intestine. The organs possess γ-glutamyl transpeptidase, an outer surface enzyme which splits GSH into cysteinylglycine and a γ-glutamyl moiety, which is transferred to an amino acid

Figure 3. Glutathione redox cycle. GSH can reduce ROS independently or, such as in the case of H_2O_2, by means of GSH peroxidase (GPx). GSH reductase (GR) subsequently reconverts GSSG to GSH at the cost of NADPH which is yielded from the pentose phosphate pathway which uses glucose-6-phosphate dehydrogenase (G6PD) to regain NADPH.



acceptor, which most often is cystine. The cysteinylglycine and γ-glutamyl cystine are subsequently transported intracellularly. Cysteinylglycine are then split into cysteine and glycine and γ-glutamyl cystine is split into γ-glutamylcysteine and cysteine. Altogether, these metabolites can be used to resynthesize GSH. If another amino acid acceptor than cystine is used, the γ-glutamyl amino acid is converted into 5-oxoproline and the corresponding amino acid. 5-oxoprolinase subsequently rediscovers glutamate.

Whereas we will focus on GSH as an antioxidant in this thesis, it is important to realize that it has a number of other metabolic functions. It is conjugated to drugs to make them more water soluble; it is involved in AA transport across cell membranes (the γ-glutamyl cycle) (47); it regulates redox-sensitive signal transduction and gene expression (48) and is involved in the rearrangement of protein disulfide bonds.

Cysteine is generally considered the rate limiting substrate for GSH synthesis. The apparent K_m values of GCL for glutamate and cysteine are 1.8 and 0.1 – 0.3 mM, respectively (49). Since the intracellular glutamate concentration is several folds higher than the K_m value of GCL for glutamate, but the intracellular cysteine concentration approximates the K_m value of GCL for cysteine, availability of cysteine most significantly influences the rate of GSH synthesis.

Vitamin C (ascorbic acid)

As opposed to most mammalian species, vitamin C cannot be synthesized by humans and should, therefore, be supplemented in the diet (50). To date, there is no universal agreement on the recommended intake. According to the Dutch Health Council, the daily requirement for 'a healthy person' is 70 mg/day. Alike GSH, vitamin C is a very potent electron donor. In addition, it reduces antioxidants such as GSH and vitamin E

from their oxidized states (51). While donating electrons, vitamin C itself is oxidized, rendering the relatively stable and ureactive ascorbyl radical and subsequently dehydroascorbic acid. Part of the dehydroascorbic acid will be recycled into vitamin C, whereas the majority will be hydrolyzed. Though vitamin C is a powerful antioxidant, it may paradoxically exhibit pro-oxidant features when administered at (extremely) high doses. This is mediated by stimulation of Fenton chemistry (52), which involves the transition metal catalyzed reduction of H_2O_2 to generate the extremely reactive and harmful $OH^{\cdot}$ radical.

Vitamin E (a-tocopherol)

In contrast with GSH and vitamin C, which are water-soluble antioxidants, vitamin E is a fat-soluble component. It acts as a preventing, chain-breaking antioxidant that scavenges lipid peroxyl radicals, thereby blocking the propagation of lipid peroxidation, while maintaining the integrity of cell membranes. During this process, vitamin E is oxidized to a tocopheroxyl radical. At this stage, it requires other antioxidants such as vitamin C to be reduced again, otherwise it will propagate the oxidative chain reaction itself. Interaction of vitamin C with the tocopheroxyl radical to regenerate vitamin E thus moves radicals from the lipid phase into the aqueous phase and thereby preventing tocopherol-mediated peroxidation (53).

Albumin

Albumin is a protein (66 kD) comprising over half of the total plasma protein pool. It has great storage capacity for both GSH and its constituent cysteine. Albumin acts as an extracellular antioxidant via its sulfhydryl groups. In addition, its heme-binding properties prevent transition metals such as iron and copper from generating the extremely reactive $OH^{\cdot}$ radicals during Fenton reactions (54, 55). Also, its presence in high quantities enables it to intercept a substantial part of the oxidant burden, providing it with passive antioxidant power.

Bilirubin

Unconjugated (indirect) bilirubin is an intracellular antioxidant that acts by binding to membranes to protect them from peroxidation. Since unconjugated bilirubin can diffuse into any cell, mild hyperbilirubinemia after birth may protect the infant during the transition from the low-oxygen intrauterine environment to the oxygen-rich extrauterine environment (56, 57).

Vitamin A, thioredoxins, enzymes such as *superoxide dismutase* or *catalase*, and trace elements such as *selenium* all have important antioxidant properties, which will, however, not be discussed in this thesis.

Oxidative Stress In Preterm Infants

Transition from the fetal to the neonatal state requires a large number of highly complex adaptations. These include expansion of the lungs with subsequently the initiation of breathing, establishment of adult type circulation, thermoregulation, and numerous metabolic adaptations. Consequently, birth may be considered physiologically the most dramatic event in life.

Due to immaturity, adequate anticipation to extrauterine life is complicated in preterm birth. In term infants, antioxidant defenses are present at birth to counteract this hyperoxic challenge, since the antioxidant enzymes mature during late gestation (58). Several weeks prior to birth, parallel with the rapid rise in lung surfactant, there is a 150-200% increase in superoxide dismutase and glutathione peroxidise as well as upregulation of other antioxidant enzymes (59-61). Also, there is an increased transfer of several antioxidants across the placenta during the last days of pregnancy, as to prepare the unborn child for the relative hyperoxia imposed by extrauterine life (58). Preterm birth, however, lacks this preparation (62, 63). In addition, due to surfactant deficiency and respiratory distress, preterm infants often require ventilatory support with supplemental oxygen, which further adds to the oxidant load. These infants are prone to develop oxidative stress.

Apart from hyperoxia, oxidative stress in preterm infants is also mediated by a number of other events. One of them is sepsis: during infection, cytokines are expressed which induce neutrophil activation followed by formation and release of ROS (64). Another, seemingly paradoxical contributor to oxidative stress is hypoxia followed by reperfusion. Hypoxia and anaerobic metabolism results in the accumulation of purine metabolites (hypoxanthine and xanthine) and accumulation of xanthine oxidase, which breaks down hypoxanthines. Activity of xanthine oxidase is limited during hypoxia since oxygen is needed to activate this enzyme. With reperfusion, oxygen availability increases rapidly, xanthine oxidase is activated, and the breakdown of accumulated hypoxanthine coincides with a burst of ROS.

In 1989, Ola Saugstad proposed the term *oxygen radical disease in neonatology* (ORDN) to describe a series of major diseases, which primarily affect the preterm newborn and were believed to share an oxidative stress mediated pathophysiology (65). This ORDN included bronchopulmonary dysplasia, retinopathy of prematurity, necrotizing enterocolitis, periventricular leukomalacia, patent ductus arteriosus, and possibly other diseases. A link has also been described between perinatal exposure to

100% oxygen and childhood cancer (66, 67). Direct evidence of an association between these diseases and oxidative stress is still lacking. Whether oxidative stress would have a causative role or is merely a direct consequence of these diseases is also unclear. A short outline of these diseases is presented below.

Bronchopulmonary dysplasia

Bronchopulmonary dysplasia (BPD), also known as chronic lung disease of prematurity, is defined as the need for supplemental oxygen at 36 weeks' postmenstrual age. Histological findings include endothelial and epithelial cell damage, bronchial smooth muscle hypertrophy, interstitial fibrosis, and simplification of the acinar structure with reduction in total number and surface area of alveoli (68).

It affects approximately 10% of VLBW infants and 40% of ELBW infants who survive to discharge (69). Although the etiology is known to be multifactorial, predisposing factors including high positive airway pressures, inflammation, pneumonia, genetic susceptibility and meconium aspiration (70), histological findings in experimental pulmonary O_2 toxicity are similar to those seen in BPD (71, 72). Indeed, ROS have been implicated in the pathogenesis of BPD (73). In addition, markers of oxidative stress are increased in infants who develop BPD (74). A number of antioxidants have been used in trials aimed at preventing BPD. Although vitamin A slightly decreased the risk of developing BPD in ELBW infants (75), vitamin E, superoxide dismutase, or N-acetylcysteine (a precursor of cysteine), did not (76-78).

Periventricular leukomalacia

Periventricular leukomalacia (PVL) results from degeneration of white matter adjacent to the cerebral ventricles following cerebral hypoxia or brain ischemia in neonates. PVL is the principal neurologic problem affecting children born extremely premature and the major pathological finding underlying cerebral palsy (79). Approximately 25% of VLBW infants who survive to discharge exhibit moderate to severe permanent motor deficits, such as spastic diplegia. The diagnosis is made by neuroimaging examinations (cerebral ultrasound and/or magnetic resonance imaging). Although its pathogenesis is multifactorial, a maturational dependent susceptibility of developing oligodendrocytes to oxidant stress seems to play an important role. Evidence was obtained using immunocytochemical markers in autopsy brain tissue of human preterm infants (80). The authors report protein nitration and lipid peroxidation in immature oligodendrocytes which was not found in control tissue. Recently, Gerstner et al. found that formation of ROS following hyperoxia triggers apoptosis in immature oligodendrocytes in the neonatal rat brain, and leads to white matter injury (81). GSH depletion aggravated

injury to immature oligodendrocytes, whereas providing substrates for GSH synthesis ameliorated oligodendrocyte injury in the developing brain (82, 83).

Retinopathy of prematurity

Retinopathy of prematurity (ROP) is a vasoproliferative disorder of the immature retina primarily affecting preterm infants. Hyperoxia and the resulting high oxygen saturation produces vasoconstriction and impairs vascularization, which leads to ischemia in certain parts of the retina, followed by vasoproliferation. During the last years, the role of VEGF, or vascular endothelial growth factor, has become apparent as a crucial factor in the pathogenesis of ROP. VEGF is inhibited by hyperoxia, which in turn leads to delayed blood vessel growth of the retina. The resulting hypoxia upregulates VEGF with uncontrolled neovascularization as a result. However, there is evidence of ROS playing part in the pathogenesis of ROP as well. In two studies, increased concentrations of hypoxanthine were found in the eyes of infants suffering from ROP (84, 85). In addition, the immature retina is deficient in most antioxidants. Papp et al. found lower plasma concentrations of GSH and selenium in infants who developed ROP as compared to control infants (86).

Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is a serious disease affecting predominantly preterm infants. Alike the other diseases presented above, which have a higher incidence, its pathogenesis is multifactorial, involving pathogenic bacterial colonization, hypoxia, and either withholding or providing too much enteral feedings in the early neonatal phase. ROS evolve following hypoxia and reperfusion as discussed earlier. Gut epithelial cells are particularly rich in xanthine oxidase, which is activated during hypoxia and reacts with oxygen during reperfusion, producing a burst of ROS. A number of animal studies have shown beneficial effects of administration of antioxidants, such as superoxide dismutase and vitamin E, in preventing intestinal damage in experimental models of NEC (87-90).

Hypotheses & outline of the thesis

This thesis describes a number of studies on early nutritional interventions in (preterm) infants and their impact on neonatal wellbeing, as reflected by protein metabolism and antioxidant defense. Note: "early nutrition" is used throughout to designate any nutri-

tional strategy that involves introduction of amino acid administration in a substantial amount (> 2 g/(kg·d)) shortly after birth (within hours).
The main hypotheses that will be tested are:

- AA administration is safe and results in anabolism, accomplished by increased protein synthesis.
- Energy for protein synthesis is derived from increased glucose oxidation.
- GSH consumption is increased in preterm infants and AA administration increases GSH synthesis rates and decreases oxidative stress.
- Cysteine availabilityadditional cysteine supplementation above a standard dosage will further increase GSH synthesis.
- Infants on ECMO and infants suffering from perinatal asphyxia experience increased oxidative stress as compared to healthy preterm infants.

Chapter 1 gives an overview of the subject of this thesis and describes current knowledge and research questions.

Part I – Amino acid administration and protein metabolism

Chapter 2 presents the results of a randomized clinical trial determining general aspects of safety and efficacy of early amino acid administration in promoting anabolism in a large group of preterm infants. In **chapter 3**, a stable isotope study, investigating the effects of early amino acid administration in preterm infants on protein synthesis, protein breakdown and glucose oxidation, is described. Whereas this chapter presents results on whole body protein kinetics, **Chapter 4** describes a study carried out to specifically investigate synthesis rates of albumin, which is the main plasma protein and an important extracellular antioxidant. **Chapter 5** presents a short report on the neurodevelopmental outcome in infants described in chapter 2 at two years of age.

Part II – Amino acid administration and antioxidant defense

Chapter 6 describes a novel method using stable isotope techniques for studying glutathione metabolism in extremely small sample volumes, such as is required for measurements in preterm infants. In **Chapter 7**, we present an observational longitudinal study in preterm infants in which we measured synthesis rates of glutathione and protein oxidation markers during the first week of life. **Chapter 8** describes a randomized clinical trial determining stimulatory effects of early amino acid administration on glutathione synthesis rates and its potential to lower oxidative stress in preterm infants. In **Chapter 9**, we describe a study investigating glutathione synthesis rates and protein damage in the immediate postnatal phase. This study was carried out to investigate whether glutathione synthesis is already stimulated within a few hours after birth. **Chapter 10** describes a randomized clinical trial which addresses the hypothesis that a high dose cysteine stimulates glutathione synthesis as compared to a lower dose in preterm infants considering cysteine is an essential amino acid in very preterm in-

fants. In **Chapter 11**, two observational studies are described on glutathione synthesis and protein damage in term infants suffering from perinatal asphyxia and term infants requiring extracorporeal membrane oxygenation (ECMO). In **Chapter 12**, the general discussion, the results of this thesis are discussed, and compared with data obtained by others. In addition, suggestions for future research are being made. Lastly, **Chapter 13** provides an overall summary.

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2 Amino acid administration to premature infants directly after birth

Frans WJ te Braake, Chris HP van den Akker, Darcos JL Wattimena, Jan GM Huijmans, and Johannes B van Goudoever

The first two authors contributed equally

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Abstract

The objective of this study was to test the hypothesis that the administration of 2.4 g amino acids (AAs)/(kg·d) to very low birth weight infants is safe and results in a positive nitrogen balance.

We conducted a randomized, clinical trial. Preterm infants with birth weights <1500 g received either glucose and 2.4 g AAs/(kg·d) from birth onward (n = 66) or solely glucose during the first day with a stepwise increase in AA intake to 2.4 g AAs/(kg·d) on day 3 (n = 69). Blood gas analysis was performed daily during the first 6 postnatal days; blood urea nitrogen levels were determined on days 2, 4, and 6; AA plasma concentrations and nitrogen balances were determined on days 2 and 4. Student *t* tests, Mann-Whitney tests, and χ^2 tests were performed to compare groups.

Infants supplemented with AAs had no major adverse side effects. Their blood urea nitrogen levels were higher, nitrogen balance turned positive upon AA administration, and more AA concentrations were within reference ranges.

In conclusion, high-dose AA administration to very low birth weight infants can be introduced safely from birth onward and results in an anabolic state.

Introduction

After birth, very low birth weight (VLBW) infants are dependent on externally administered nutrients, as hardly any stored energy is at their disposal (1). Both fat tissue and glycogen levels are limited, especially in small for gestational age (SGA) VLBW infants. Consequently, without adequate exogenous nutrient supply, protein breakdown will increase in these infants, resulting in a catabolic state.

Despite a growing body of literature regarding the safety and efficacy of early amino acid (AA) administration, there is still wide variability in practice. Often, carbohydrates are still the only exogenous nutrients administered in the immediate postnatal period. In the past, AAs were often withheld since formerly used AA mixtures were found to result in metabolic acidosis and hyperammonemia (2, 3). In utero, fetuses are supplied with large amounts of AAs, which not only are used for protein synthesis but also serve as an important fuel source (4-7). It seems logical, therefore, to supply newborn infants with adequate amounts of both energy and growth substrates to meet energy requirements and to promote protein accretion for ongoing growth. Indeed, several studies indicate that the currently used crystalline solutions seem well suited for the preterm infant, who may benefit from the anabolic effects (8-14). However, in most of these studies, either low amounts of AAs were administered, administration started only after the first day of life, infants with higher birth weights were studied, or the number of infants studied was small.

Hypothesizing that premature infants may benefit from the anabolic effects of AAs without metabolic derangement, we investigated the safety and efficacy of relatively large amounts of AAs supplied postnatally to a large group of VLBW infants.

Methods

A randomized, blinded trial was performed in the neonatal intensive care unit (NICU) of the Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands. For logistic reasons, it was not possible to perform the study using a double-blinded fashion. The trial was investigator-initiated, with no funding from the pharmaceutical industry. The study protocol was approved by the Erasmus MC Medical Ethical Committee, and parental consent was obtained before random assignment and subsequent enrollment in the study.

Study Design

Prematurely born infants with birth weights equal or less than 1500 g born between March 2003 and September 2004 in the hospital and admitted to the NICU were randomly assigned to receive one of two parenteral nutritional schemes, as indicated in Table I. The amount of 2.4 g AAs/(kg·d) was chosen because that was the amount that resulted in a positive nitrogen balance in an earlier study (14).

Table I: Targeted intravenous macronutrient intake in mg/(kg·min) (glucose) or g/(kg·d) (AAs and lipids).

		Day			
		1	2	3	4
Intervention	Glucose	5.5	5.6	5.7	7.1
	AAs *	2.4	2.4	2.4	2.4
	Lipids †	0	1.4	2.8	2.8
Control	Glucose	5.5	5.6	5.7	7.1
	AAs *	0	1.2	2.4	2.4
	Lipids †	0	1.4	2.8	2.8

If enteral feedings were tolerated, parenteral glucose intake was decreased.

* Primene 10%, Baxter, Clintec Benelux NV, Brussels, Belgium.

† Intralipid 20%, Fresenius Kabi BV, 's Hertogenbosch, the Netherlands.

After the third day of life, all nutrient intakes, including enteral feedings, were the decision of the attending neonatologist. Minimal enteral nutrition (6 to 12 feedings of 1.0 mL) was whenever possible started on postnatal day 2 to day 3 and advanced to full enteral nutrition in the subsequent days if tolerated. We recorded birth weight, gestational age, percentage of SGA infants (<−2 SD) (15), sex ratio, number of prenatal corticosteroid doses (0, 1, or 2), and severity of illness at entry to the study with Apgar and CRIB scores (16). Exclusion criteria were known congenital abnormalities, chromosome defects, metabolic diseases, and endocrine, renal, or hepatic disorders.

Analysis

Safety

We analyzed blood gas and glucose concentrations 12 hours after delivery, followed by daily measurements at 8 am until day 6. Blood urea nitrogen (BUN) concentrations were monitored on days 2, 4, and 6 (Roche Hitachi 912, Roche Diagnostics, Basel, Switzerland). On days 2 and 4, we determined plasma AA concentrations (Biochrom 20,

Biochrom Ltd, Cambridge, England) in a subset of patients (intervention group n = 17, control group n = 14) to identify possible hyperaminoacidemia (ie, above reference ranges, as defined in Reference 22). We also recorded fluid intakes and medications.

Efficacy

Efficacy of early AA administration was studied by quantifying the nitrogen balance in both groups on postnatal days 2 and 4. Because most nitrogen leaves the body in urine, we collected urine during a 12-hour period on both study days. Urinary nitrogen content was measured with a CHN elemental analyzer (ANA 1500; Carlo Erba Strumentazione, Milan, Italy). By subtracting the calculated nitrogen excretion rates from the precisely recorded nutritional intakes, nitrogen balances could be defined under the assumption that 1 g of nutritional AAs equals 160 mg of nitrogen. Although 24-hour collections of urine are preferable, 12-hour or even 6-hour collections can be used to establish reasonable estimates of nitrogen excretion (17). Many investigators used 12-hour urine collections accordingly (8, 11, 12, 18). Finally, to express efficacy in terms of a measurable clinical variable, we recorded on which postnatal day infants regained their birth weight.

Statistics

Differences between groups were tested by Student *t* tests, Mann-Whitney tests, and χ^2 tests, using SPSS version 11.0 (SPSS Inc, Chicago, IL). Depending on distribution and type of test, values are expressed as mean ± SD, as median (min-max), or as percentage, respectively. Significance level was set at $P < .05$. However, because of multiple variables assessed on single samples, differences in AA concentrations were considered to be statistically significant at $P < .01$. From previous findings, we calculated that with a power of 0.80, group size needed to be at least 26 to detect a difference in the nitrogen balance of 150 mg N/(kg·d), with a standard deviation of 120 mg N/(kg·d). However, as we intended to study safety aspects as well, we continued to include patients for the full 18 months.

Results

We included 66 infants in the intervention group and 69 in the control group; all infants were included on the basis of intention to treat (Table II). Despite random assignment, infants in the intervention group were more frequently exposed to prenatal corticosteroids (P = .017). According to study design, the infants in the intervention group

Table II: **Clinical characteristics of the infants in the intervention and control group.**

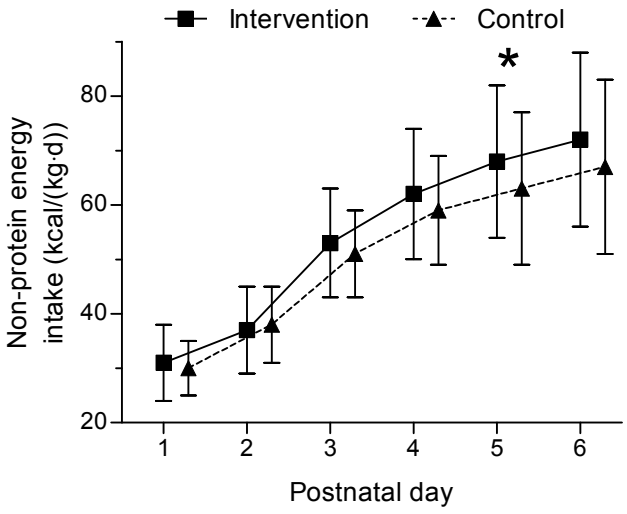
	Intervention	Control
N (male/female)	66 (34/32)	69 (31/38)
Birth weight (g) *	1039 ± 235	989 ± 252
Gestational age (wk) *	28.4 ± 2.0	28.4 ± 1.9
SGA infants (<−2 SD)	20%	29%
CRIB score †	3 (0–13)	4 (0–14)
Apgar (5') score †	9 (1–10)	8 (2–10)
Prenatal corticosteroids (% 0/1/2 doses)	18/18/64	39/19/42

* Values are expressed as mean ± SD.

† Values are expressed as median (min-max).

received AA within 2 hours after birth (median, 1 hour, 33 minutes). Nonprotein energy intakes did not differ between groups, except on day 5 (68 ± 14 [intervention] vs 63 ± 14 [control] kcal/[kg·d]; P = .033) (Figure 1).

Figure 1. **Total nonprotein energy intakes (parenteral and enteral) during the first postnatal week. * Statistically significant; P < 0.05 (Student t test).**



Safety

Results of blood gas analysis and whole blood glucose levels 12 hours after birth and on the second day are shown in Table III. Between postnatal days 3 and 6, there were no differences. BUN levels are shown in Table IV.

Table V shows individual plasma AA concentrations on the second day of life. No statistical differences between the two groups were found on the fourth postnatal day.

Medications, including sodium bicarbonate for metabolic acidosis, were not different between groups.

Table III: Blood gas analysis and whole blood glucose concentrations in the intervention and control groups 12 hours postnatally and on postnatal day 2.

	12 h		Day 2	
	Intervention	Control	Intervention	Control
pH	7.33 ± 0.08	7.34 ± 0.08	7.31 ± 0.06	7.32 ± 0.07
BE (mmol/L)	−4.8 ± 3.1	−3.7 ± 3.3	−5.7 ± 2.4 *	−4.4 ± 2.4
Bicarbonate (mmol/L)	20.5 ± 2.6 *	21.5 ± 2.6	20.3 ± 2.5 *	21.4 ± 2.2
Glucose (mmol/L)	5.7 ± 3.2	6.1 ± 2.4	4.4 ± 1.9 *	5.3 ± 2.1

Values represented as mean ± SD and tested with Student t test.

* Statistically significant; P < 0.05.

Table IV: BUN levels in mmol/L and (mg/dL), respectively on postnatal days 2, 4, and 6.

	Intervention	Control
day 2	9.6 ± 2.8 (27.0 ± 7.8) *	6.0 ± 1.8 (16.7 ± 5.2)
day 4	9.4 ± 3.5 (26.4 ± 9.8) *	6.0 ± 3.3 (16.8 ± 9.2)
day 6	8.4 ± 3.8 (23.6 ± 10.7) *	6.7 ± 3.1 (18.7 ± 8.7)

Values represented as mean ± SD.

* Statistically significant; P < 0.05.

Efficacy

As follows from study design, nitrogen intake on the second day was higher in the intervention group (Figure 2). On the fourth day, intakes were similar between groups. Nitrogen excretion rates in the intervention group exceeded excretion rates in the control group on both day 2 and day 4. Furthermore, within the intervention as well as within the control group, rates of excretion did not change between days 2 and 4. Consequently, nitrogen balance was higher in the intervention group on day 2 as compared with the control group, which had a negative nitrogen balance. On the fourth

Table V: Plasma AA concentrations in the intervention and control groups on postnatal day 2 (mean ± SD) and reference values from healthy term breast-fed infants on postnatal day 11 (reference 22). Values are expressed as µmol/L.

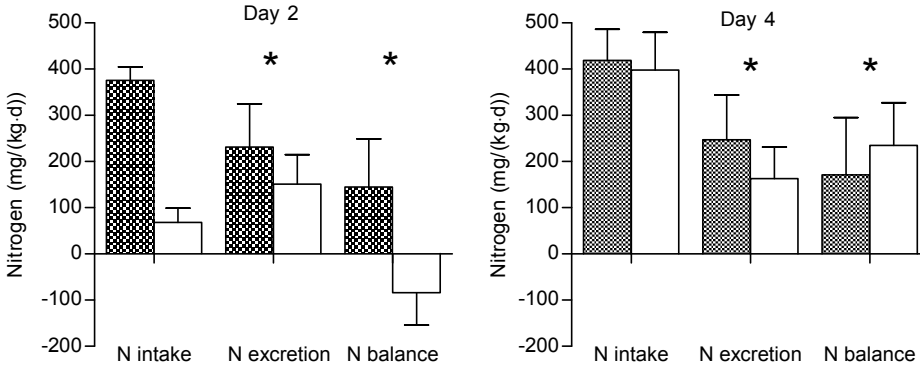
	Intervention	Control	Reference range
Leucine **	148 ± 43	47 ± 13	86 – 171
Isoleucine **	88 ± 33	18 ± 8	31 – 124
Valine **	281 ± 90	88 ± 23	56 – 154
Threonine	125 ± 48	123 ± 63	67 – 143
Lysine **	345 ± 144	98 ± 34	65 – 282
Histidine **	103 ± 53	52 ± 19	25 – 126
Methionine *	42 ± 22	22 ± 9	21 – 55
Phenylalanine **	92 ± 31	58 ± 10	35 – 112
Cystine	31 ± 79	22 ± 12	33 – 55
Tyrosine	83 ± 43	122 ± 57	48 – 122
Alanine **	265 ± 139	124 ± 67	137 – 362
Proline *	175 ± 89	102 ± 56	
Serine *	186 ± 89	116 ± 49	79 – 227
Glycine	282 ± 161	205 ± 70	66 – 432
Arginine **	70 ± 19	29 ± 12	11 – 88
Glutamine	507 ± 296	313 ± 153	147 – 623
Glutamate **	64 ± 34	22 ± 9	76 – 551
Asparagine	39 ± 23	49 ± 24	16 – 21
Aspartate *	35 ± 16	18 ± 14	5 – 46
Taurine	150 ± 87	106 ± 112	
Citrulline	54 ± 67	31 ± 44	20 – 84
Ornithine **	180 ± 87	40 ± 13	39 – 386
OH-Proline	47 ± 26	46 ± 28	

* Statistically significant; $P < 0.01$.

** Statistically significant; $P < 0.001$.

day, nitrogen balances in both groups were positive. However, in the control group, the balance was more positive than in the intervention group. There was no correlation between antenatal steroid administration and nitrogen excretion or balance. Fluid intakes were higher in the intervention group on both postnatal day 1 and day 2 due to the administration of AA. On all other days, fluid intakes were similar. Fluid balances, determined on postnatal days 2 and 4, did not differ between groups. Age to regain

Figure 2. Nitrogen balances on postnatal days 2 and 4. * Statistically significant; P < 0.05 (Student t test). Hatched bars represent the intervention group; open bars the control group.



birth weight was not statistically different; newborn infants in the intervention group regained their birth weight at day 8 (2-25) (median and [min-max]), those in the control group at day 10 (2-26) (P = 0.286).

Discussion

The currently available AA solutions are safe and can be administered to premature infants during the first few days of life (8-14). We performed the largest study to date confirming the safety and anabolic effects of early AA administration beginning within 2 hours after birth. Unlike most other reports, we did find modestly altered blood gas values and increased BUN levels with early AA administration. This could be due to the inclusion of fewer infants in other studies, with subsequently the possibility of reduced statistical power. Another explanation could be the early start of AA administration in our study, which was within 2 hours instead of 24 hours after birth (10, 12) or even later (14). In addition, others used a smaller amount of AAs (≤1.5 g/[kg·d]) (8, 13) or included infants with higher birth weights (13, 14).

We found that early AA administration normalized the plasma concentrations of most AAs and that nitrogen balance was positive on day 2 of life, despite a relatively low energy intake (<40 kcal/[kg·d]). BUN levels were higher in the intervention group, which theoretically could have increased urine production but in fact did not (data not shown). Besides, fluid balance is usually tightly controlled in NICUs. To our knowledge, no other potential side effects of increased BUN levels have been reported. The higher BUN levels are a reflection of a higher AA oxidation rate. This resembles the intrauterine situation in which AA seem to be a key nutrient for energy generation (5, 7) and where

BUN reference values for human umbilical cord blood are 7.5 to 14.3 mmol/L (21.0 to 40.1 mg/dL) (19).

In conjunction with the higher BUN levels, the higher amounts of excreted nitrogen in the intervention group also indicate a higher oxidation rate. Higher BUN levels should, therefore, not be interpreted as a sign of AA intolerance but rather as a reflection of AA oxidation, just like in utero, where the AAs are partly oxidized and partly used for protein synthesis.

Many of the infants in the intervention group had on average less hyperglycemia than did the control group, which might be explained by higher insulin concentrations triggered by relatively higher plasma arginine and leucine concentrations (12, 20, 21). In addition to these two AAs, all essential AA levels, except for threonine and most of the nonessential AA concentrations, were higher and were within the reference range in the intervention group on the second day of life (22). Although the plasma concentrations of valine, lysine, and asparagine exceeded the reference values measured postnatally in term breast-fed infants, the former two AA concentrations fit within intrauterine reference ranges (23).

The nitrogen balance was calculated by subtracting nitrogen excretion from nitrogen intake. However, nitrogen excretion is often modestly underestimated, because of incomplete urine collections and stool, breath, and skin losses, which are not accounted for (24). Furthermore, although nitrogen balance measurements demonstrate net loss or accretion of protein, they do not reveal the mechanisms underlying these conditions. Previously performed studies using stable isotope techniques showed that premature infants supplied with AAs have an improved balance, which is due to increased protein synthesis, while proteolysis is not suppressed (8, 12, 14, 25).

Inasmuch as premature infants cannot survive without growth, we conclude that the administration of AAs soon after birth with the aim of promoting anabolism is safe and effective.

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3 Effects of early amino acid administration on leucine and glucose kinetics in premature infants

Chris HP van den Akker, Frans WJ te Braake, Darcos JL Wattimena, Gardi Voortman, Henk Schierbeek, Andras Vermes, and Johannes B van Goudoever

The first two authors contributed equally
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Abstract

We previously showed that, in prematurely born infants, an anabolic state without metabolic acidosis can be achieved upon intravenous amino acid (AA) administration in the immediate postnatal phase, despite a low energy intake. We hypothesized that the anabolic state resulted from an increased protein synthesis and not a decreased proteolysis. Furthermore, we hypothesized that the energy needed for the higher protein synthesis rate would be derived from an increased glucose oxidation. To test our hypotheses, 32 ventilated premature infants (<1500 g) received intravenously either solely glucose or glucose and 2.4 g AAs/(kg·d) immediately postnatally. On postnatal day 2, each group received primed continuous infusions of either [1-^{13}C]leucine or [U-$^{13}C_6$]glucose. $^{13}CO_2$ enrichments in expiratory air and plasma [1-^{13}C]α-KICA (as an intracellular leucine precursor) and [U-$^{13}C_6$]glucose enrichments were measured by mass spectrometry techniques. The AA administration resulted in an increased incorporation of leucine into body protein and a higher leucine oxidation rate, whereas leucine release from proteolysis was not affected. Glucose oxidation rate did not increase upon AA administration. In conclusion, the anabolic state resulting from AA administration in the immediate postnatal period resulted from increased protein synthesis and not decreased proteolysis. The energy needed for the additional protein synthesis was not derived from an increased glucose oxidation.

Introduction

A series of studies on AA administration in premature infants within the first few postnatal days show a positive effect on nitrogen retention or plasma AA concentrations starting immediately after birth (1, 2), within or at 24 h postnatally (3-5), or later (6). In our latest study regarding early AA administration, we administered 2.4 g AAs/(kg·d) to one half of 136 VLBW infants within 2 h postnatally (1). This resulted in a positive nitrogen balance and converted plasma AA concentrations to levels fitting reference ranges. Furthermore, there were no major metabolic disturbances in comparison with the group receiving solely glucose.

However, nitrogen balance calculations provide no information on how a particular nutritional status was reached. An anabolic state can arise from increased protein synthesis, decreased protein breakdown, or a combination of both. To clarify the mechanism by which an anabolic state is reached in VLBW infants, we conducted in a first trial a stable isotope study using L-[1-^{13}C]leucine. We speculated that the anabolic state would have been induced by an increased protein synthesis, a phenomenon also observed in other studies, none of which, however, started AA supplementation immediately after birth (3, 5, 6). Furthermore, by collecting $^{13}CO_2$ we were able to quantify leucine oxidation rates.

We hypothesized that the extra energy required for protein synthesis would be derived from additional glucose oxidation. Therefore, we studied glucose metabolism in a second trial in which infants also received either solely glucose or glucose and AAs, using D-[U-$^{13}C_6$]glucose as a tracer.

Methods

The included infants were a subset of the patients included earlier by Te Braake et al. (1) in a study determining safety and efficacy of high-dose early AA administration. The present study was designed as a randomized open trial and was performed in 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 protocol was approved by the Erasmus MC Medical Ethical Committee and parental consent was obtained before the study.

Patients

Thirty-two prematurely born infants with a birth weight <1500 g, who were born in the Erasmus MC – Sophia Children's Hospital, were mechanically ventilated, had an arterial

catheter, and were expected to be completely dependent on parenteral nutrition for the first 2 d of life, were directly after birth randomly assigned to receive either i) only glucose during the first 2 d (control group, n = 16) or ii) glucose and 2.4 g of protein/(kg·d) as AAs (Primene 10%, Baxter, Clintec Benelux N.V., Brussels, Belgium) within 2 h postnatally (intervention group, n = 16).

AA and/or glucose solutions were constantly infused without interruptions during the study. Lipids and/or (minimal) enteral feedings were not administered until after the study period. Exclusion criteria were known congenital abnormalities, chromosome defects, and metabolic, endocrine, renal, or hepatic disorders. For all infants, we recorded birth weight, gestational age, SD scores for weight (7), antenatal corticosteroid usage, and severity of illness at entry of the study by means of Apgar and CRIB scores (8). We also assessed blood gases and nitrogen balances as described previously (1).

The control and intervention groups were each subdivided into two cohorts (n = 8 each). In one cohort (A), we studied the effects of early AA administration on leucine kinetics on postnatal d 2. In the other cohort (B), we determined glucose kinetics on d 2 upon early AA administration.

Stable isotopes

[^{13}C]Sodium bicarbonate ($NaH^{13}CO_3$) (99% enriched), L-[1-^{13}C]leucine (99% enriched), and D-[U-$^{13}C_6$]glucose (99% enriched) were purchased from Cambridge Isotope Laboratories (Andover, MA) and were diluted with a 0.9% saline solution by the hospital's pharmacy after which it was tested on sterility and pyrogenicity. For the leucine study, the bicarbonate pool was initially enriched with a primed (10 μmol/kg) continuous $NaH^{13}CO_3$ infusion (10 μmol/(kg·h)). After 2 h, the infusion was replaced by a primed (15 μmol/kg) continuous L-[1-^{13}C]leucine infusion (15 μmol/(kg·h)) lasting for 5 h (Figure 1**A**).

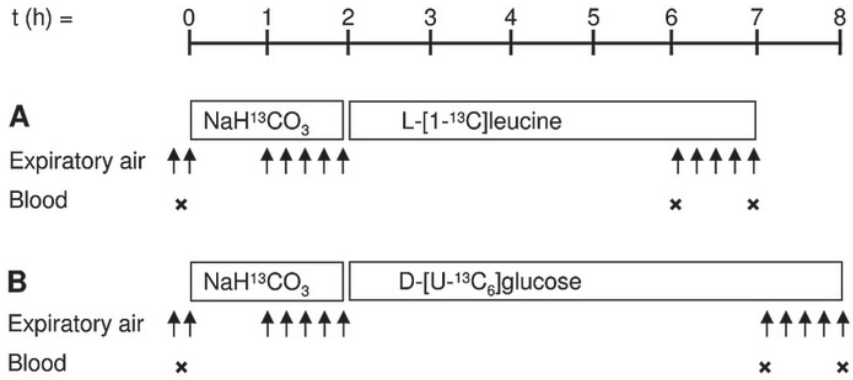
In the second cohort (the glucose study), the bicarbonate pool was also enriched with a primed (15 μmol/kg) continuous $NaH^{13}CO_3$ infusion (15 μmol/(kg·h)). After 2 h, the infusion was replaced by a primed (10 μmol/kg) continuous D-[U-$^{13}C_6$]glucose infusion (5 μmol/(kg·h)) lasting for 6 h (Figure 1**B**).

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.

Measurement of isotopic enrichments in plasma

Arterial blood samples were drawn once before the isotope infusions (baseline) and twice during the last hour of the leucine or glucose tracer infusion. After collection, the samples were put on melting ice immediately and centrifuged, after which the plasma was aspired and stored at -80°C until analysis.

Figure 1. **Study design. Infants in both the control and intervention group were subjected to either the labeled leucine (A) or the labeled glucose (B) protocol on postnatal day 2.**



Within the cell, leucine is reversibly transaminated to its keto-analogue, α-KICA. The plasma enrichment of [1-^{13}C]α-KICA is very close to intracellular [1-^{13}C]leucine enrichment. Measurement of the enrichment of [1-^{13}C]α-KICA after L-[1-^{13}C]leucine infusion will, therefore, reflect both the site of incorporation of leucine in protein and the site for the irreversible decarboxylation of [1-^{13}C]α-KICA to isovaleryl-CoA and $^{13}CO_2$ (9, 10). Samples (50 µL plasma) were treated and analyzed as previously described (2, 11). The ^{13}C enrichment of α-KICA was, after derivatization to butyldimethyl-silylquinoxalinol derivatives, determined with a Carlo Erba GC8000 gas chromatograph coupled to a Fisons MD800 mass spectrometer (Interscience BV, Breda, the Netherlands) by measuring the intensity of the 259 and 260 fragments in electron impact ionization mode.

The [U-$^{13}C_6$]glucose enrichment of the glucose aldonitril pentaacetate derivatives was monitored, after combustion to carbon dioxide at mass 44 for $^{12}CO_2$ and mass 45 for $^{13}CO_2$, using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C-IRMS) (Delta XP, Thermo Electron, Bremen, Germany).

Oxidation measurements

To determine the fractions of leucine or glucose oxidized, approximately 15 mL of expiratory air was collected in a vacuum tube at the outlet of the ventilator: two times in duplicate before the isotope infusion (baseline), five times in duplicate during the last hour of the $NaH^{13}CO_3$ infusion, and five times in duplicate during the last hour of the labeled leucine or glucose infusion. We assumed an equal CO_2 production and retention during the sodium bicarbonate and leucine or sodium bicarbonate and glucose infusions. Breath samples were analyzed for $^{13}CO_2$ enrichment on an isotope ratio mass spectrometer (IRMS) (ABCA, Europe Scientific, Van Loenen Instruments, Leiden, the Netherlands).

Calculations

The turnover rates were calculated by measuring tracer dilution in plasma at steady state with standard isotope equations, as previously described for leucine (2) and glucose (12) studies.

Statistics

Based on previous findings from our study group, we calculated that with an α of 0.05, a power of 0.80, and a difference in protein synthesis rate of 1.4 g/(kg·d) with an SD of 0.8, group size in the leucine study needed to be at least six to detect a difference (6). A statistically detectable increment in glucose oxidation of 2.0 mg/(kg·min) with an SD of 1.0 would also require six infants in each group to be studied (12). However, both in the leucine and the glucose studies, we included eight infants in the intervention and control groups and the control groups to increase power. One-way ANOVA was used to detect differences between group characteristics, clinical laboratory measurements, and nutritional intakes between the four subgroups. Differences between intervention and control groups were tested by t-tests, Mann-Whitney tests, and χ^2 tests, as appropriate, using SPSS version 11.0 (SPSS Inc., Chicago, IL). Depending on distribution and type of test, values are expressed as mean ± SD, as median (25^{th} to 75^{th} percentile), or as percentage, respectively. Significance level was set at $p < 0.05$.

Results

We included 32 infants, of whom 7 were small for gestational age (<−2 SD) (7). Patient characteristics are provided in Table I. Overall, the infants in the intervention group had,

Table I: Clinical characteristics of the infants in the control and intervention groups.

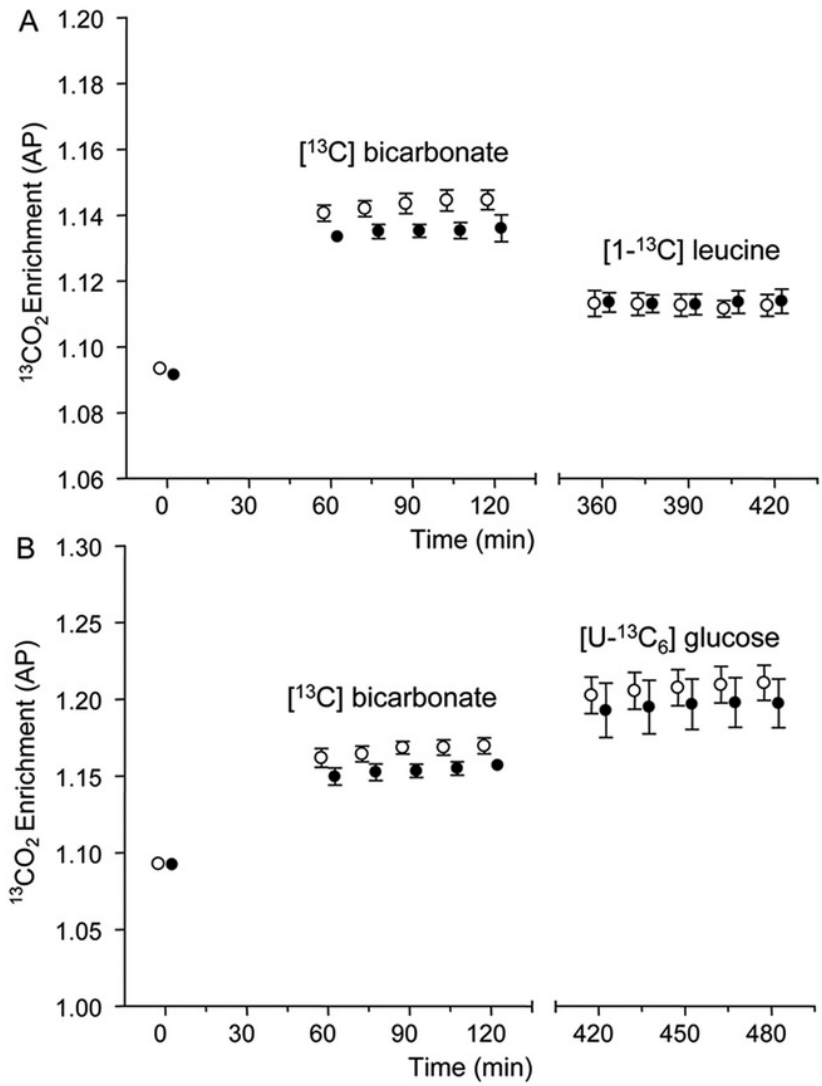
CRIB is clinical risk index for babies (8).

	Control	Intervention
N (male/female)	16 (9/7)	16 (8/8)
Birth weight (kg) *	0.949 ± 0.231	0.923 ± 0.192
Gestational age (wks) *	27.4 ± 1.4	27.3 ± 1.8
SD score for weight *	-0.9 ± 1.4	-1.0 ± 1.3
CRIB score **	6 (3-9)	5 (2-7)
Apgar score (5 min) **	8 (7-9)	9 (8-9)
Antenatal corticosteroids (0/1/2 doses)	8/6/2	2/4/10

* Values are expressed as mean ± SD. ** Values are expressed as median (25th – 75th percentile).

by coincidence, received antenatal steroids more often than those in the control group. AA administration to the infants in the intervention group started within 2 h postnatally. The stable isotope study was started on the second postnatal day, i.e., between 20 and 44 h after birth. Isotopic steady state in $^{13}CO_2$ excretion in expiratory air was reached in all infants during the last hour of each infusion (Figure 2). The actual protein intakes at time of study were 0 ± 0 and 2.32 ± 0.08 g/(kg·d) ($p < 0.001$) and the nonprotein

Figure 2. $^{13}CO2$ excretion curve during the leucine (A) and glucose (B) experiment. Enrichment is represented in atom percent (AP) as mean ± SD in the control (open circles) and intervention (filled circles) groups.



energy intakes (solely glucose) were 34 ± 8 and 30 ± 6 kcal/(kg·d) (p = 0.10) in the control and intervention groups, respectively. Other relevant patient data are provided in Table II.

Table 2: Study parameters on the second day of life in the control and intervention groups. Values depicted as mean ± SD, except for glucose concentration, i.e. median (25th – 75th percentile).

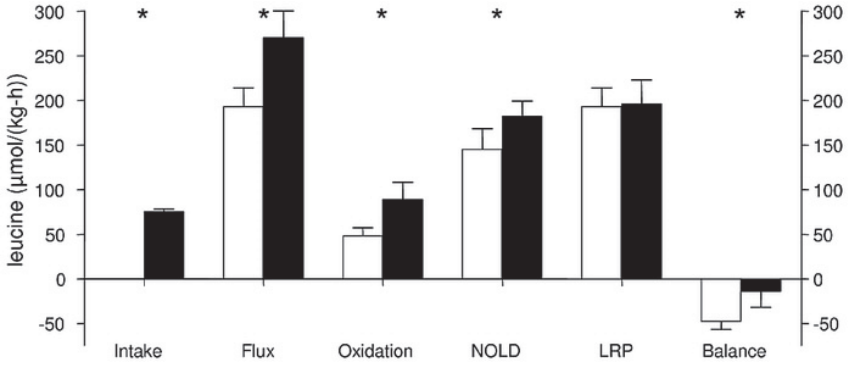
	Control	Intervention
glucose intake (mg/(kg·min))	5.9 ± 1.3	5.2 ± 1.0
glucose concentration (mmol/L)	6.1 (4.2 – 6.9)	4.6 (3.7 – 5.4)
Nitrogen balance (mg N/(kg·d))	-99 ± 42	151 ± 105 *
pH	7.31 ± 0.05	7.30 ± 0.07
Base Excess (mmol/L)	-4.4 ± 1.3	-5.4 ± 2.0
Blood urea nitrogen (mmol/L)	6.2 ± 1.5	9.7 ± 2.6 *

* Statistically significant; p<0.05.

Inasmuch as we only performed a power calculation on protein synthesis and glucose oxidation rates, all other outcomes should be regarded as hypothesis-generating data.

Leucine kinetic data are displayed in Figure 3. Infants in the intervention group had a higher leucine flux, NOLD rate (indicative of protein synthesis), and oxidation rate. The LRP rate (indicative of protein breakdown) was not altered. Leucine balance improved

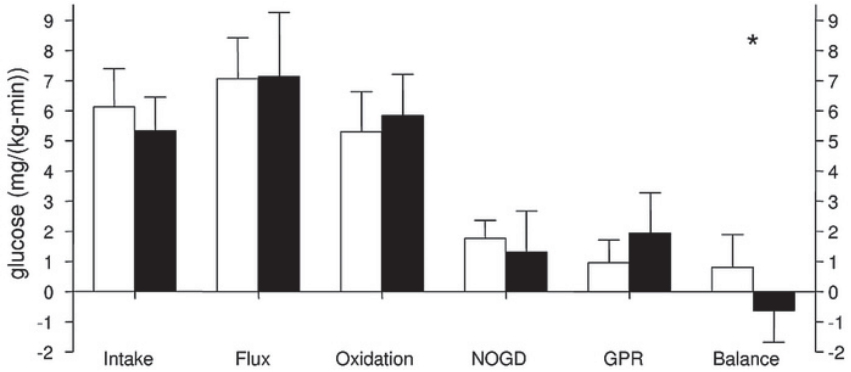
Figure 3. Leucine kinetics. Data from the [1-13C]leucine infusion protocol (A) on postnatal d 2 in infants in the control (open bars, n = 8) and intervention (filled bars, n = 8) groups. Bars represent mean ± SD. NOLD represents protein synthesis. LRP represents protein breakdown. *Statistically significant, p < 0.05.



significantly in the infants receiving AAs ($p < 0.001$). The control group had a negative balance, whereas the balance in the intervention group was not different from zero.

The glucose kinetic data are outlined in Figure 4. AA administration did not have any significant effects on glucose metabolism with regard to GPR, flux, oxidation, and NOGD

Figure 4. Glucose kinetics. Data from the [U-$^{13}C_6$]glucose infusion protocol (B) on postnatal d 2 in infants in the control (open bars, n = 8) and intervention (filled bars, n = 8) groups. Bars represent mean ± SD. *Statistically significant, p < 0.05.



rate.

In both groups, GPR (gluconeogenesis or glycogenolysis) was not completely inhibited, despite a mean intake of 5.7 ± 1.2 mg glucose/(kg·min) during the study period. In the control group, 75% of the glucose flux was oxidized; in the intervention group, this fraction amounted to 84% ($p = 0.19$). The absolute amount of oxidized glucose did not differ significantly between groups (5.3 ± 1.3 mg/(kg·min) and 5.8 ± 1.4 mg/(kg·min), respectively; $p = 0.46$).

Discussion

We found that AA administration at a relatively high dose from birth onward exerted its anabolic effect through increased protein synthesis and not decreased proteolysis. The additional energy needed was not derived from glucose, but could, at least partially, be derived from a concomitant increase in AA oxidation.

Most studies in premature infants, including the present, show a positive effect of AA administration on protein accretion caused by an increased synthesis rate (3, 5, 6, 13) . Also, in the ovine fetus, AA administration does have a beneficial effect on protein accretion by increasing protein synthesis while leaving proteolysis unaltered

(14). One study, however, investigating a short-term change in nutritional regimen, found a simultaneous decrease in proteolysis (15).

Anabolism in adults (16, 17) and healthy term infants (18, 19), unlike in preterm infants and ovine fetuses, is predominantly achieved by suppression of proteolysis instead of protein synthesis. Possibly, a new balance between protein breakdown and synthesis is developing during early life, explaining this observed discrepancy.

We found a positive nitrogen balance, not only in the intervention group of the 135 infants studied earlier (1), but also in the intervention group of the leucine cohort in this study. However, the leucine balance was not significantly different from zero. Nevertheless, there is still a significant correlation between both balance methods (r^2 = 0.47, p = 0.003). This discrepancy, which we and others noted before (2, 3), might be explained by the relative abundance of leucine in parenteral AA solutions relative to the occurrence of leucine in body protein. Because the rate of protein deposition is controlled by the rate-limiting AA in the AA solution, all excess AAs are oxidized. Leucine in particular might be oxidized pro rata more than other AAs, explaining a negative leucine balance despite a positive nitrogen balance. On the other hand, the difference between the stable isotope and nitrogen balance techniques could also be partially due to the tendency to overestimate nitrogen retention (20).

In our study, early AA administration had hardly any effect on glucose metabolism. Glucose oxidation, NOGD, and GPR were unaltered. In adults, AA supplementation, and thus provision of gluconeogenic substrates, was found to result in an increased endogenous GPR (21). This contrasts with findings in premature neonates from the study by Poindexter et al. (13) and the present study. Like in adults, we expected to find a higher glucose oxidation rate for generating the energy needed for extra protein synthesis after AA administration. Poindexter et al. (13), using indirect calorimetry, also suggested a higher glucose oxidation rate after AAs had been introduced, inasmuch as the respiratory quotient increased. Surprisingly, we could not detect any difference in oxidation rate. The source of the needed extra energy remains, therefore, speculative. Nevertheless, the higher leucine oxidation rate might reflect that AA oxidation itself might provide some of the energy needed. Other sources of energy could include fatty acids and ketone bodies.

In conclusion, AA administration to premature infants from birth onward reverses the catabolic state that is otherwise obtained when AAs are withheld. Particularly protein synthesis rate especially is increased. The additional energy needed for this process is not derived from glucose oxidation.

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4 Albumin synthesis in premature neonates is stimulated by parenterally administered amino acids during the first days of life

Chris HP van den Akker, Frans WJ te Braake, Henk Schierbeek, Trinet Rietveld, Darcos JL Wattimena, Jan Erik H Bunt, and Johannes B van Goudoever

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Abstract

Recently, we demonstrated that parenteral administration of amino acids (AAs) immediately after birth to premature infants is safe and results in a positive nitrogen balance and increased whole body protein synthesis. However, we did not determine organ specific effects. Albumin, produced by the liver, is an important protein, but its concentration is often low in premature neonates during the first few days after birth.

Our objective was to test the hypothesis that the albumin fractional and absolute synthesis rates would increase upon AA administration following birth, even at low nonprotein energy intake.

Ventilated premature infants (<1500 g) received from birth onwards either solely glucose (control group, n=7) or glucose and 2.4 g AAs/(kg·d) (intervention group, n=8). On postnatal day 2, all received a primed continuous infusion of [1-^{13}C]leucine and mass spectrometry techniques were used to determine the incorporation of the leucine into albumin. Results are expressed as median (25^{th} – 75^{th} pctl).

Albumin fractional synthesis rates in the intervention group were significantly higher than those in the control group (22.9 (17.6 – 28.0) %/d versus 12.6 (11.0 – 19.4) %/d; p=0.029). Likewise, the absolute synthesis rates in the intervention group were higher than those in the control group (228 (187 – 289) mg/(kg·d) versus 168 (118 – 203) mg/(kg·d); p=0.030).

In conclusion, AA administration increases albumin synthesis rates in premature neonates even at low energy intake.

Introduction

Plasma albumin concentration is a routinely measured parameter on the neonatal intensive care unit (NICU) and is often found to be low in ill premature infants (1, 2). Albumin, produced by the liver, has several important roles in neonatal physiology (3, 4). It is the main preserver of the colloid osmotic pressure in plasma (~75%), functions as an anticoagulant, and is an important binding transporter of certain metabolites, e.g. bilirubin, free fatty acids, and drugs. Moreover, albumin is an important antioxidant because it has specific binding sites for copper ions and a free sulfhydryl group, which can scavenge harmful reactive oxygen species (5). The free sulfhydryl group can also bind nitric oxide (NO) forming a reservoir for this regulator of vascular tonus (6). Furthermore, albumin synthesis probably provides for temporary 'storage' of amino acids (AAs) so as to spare them from oxidation (7-9). Albumin consists of 585 AA and is the most abundant plasma protein (2), though about 60% of the total albumin pool is in the interstitial space (10).

Albumin metabolism has been studied mainly in healthy adults and in adults during various stages of renal or liver disease. Most studies in neonates are limited to static properties such as concentrations. Measuring albumin synthesis rates would no doubt provide more insight into the dynamics of albumin metabolism and its response to nutrition, for example.

Several authors have described relations between low albumin concentration and morbidity and mortality rates among premature neonates (11, 12). In the fasting state, albumin concentrations drop 2 to 3 g/L in the first 24 hours after birth (2). As there is discussion about benefit and safety of exogenous albumin infusions in premature infants (13-15), stimulating endogenous synthesis via adequate nutritional support might be an attractive alternative.

The latter strategy requires good knowledge of premature infants' protein metabolism. Studies using stable isotopes have provided insights into anabolism and catabolism in general (16-18). For one, AA administration directly after birth stimulates whole body protein synthesis rather than depressing protein breakdown (16). Studying whole body metabolism is limited, however, in that it only provides information on the average of all metabolic processes in the body rather than organ specific changes.

It is unknown whether exogenous administration of AAs also stimulates organ specific protein synthesis, e.g. albumin, in premature infants. Albumin synthesis can be quantified by measuring the incorporation rate of a stable isotope labeled AA into plasma albumin.

We report a study in preterm infants aimed at determining the effect of AA administration starting directly after birth on subsequent albumin synthesis. We hypothesized that AAs added to glucose would increase albumin synthesis rates.

Methods

Subjects

Patients were eligible for the study when they were in the Sophia Children's Hospital, had a birth weight less than 1500 g, and required an arterial line for clinical purposes. Exclusion criteria were known congenital abnormalities, chromosome defects, and metabolic, endocrine, renal or hepatic disorders. The cohort here described is the same as in an earlier study on whole body leucine metabolism (16). It is a subset of infants participating in a large trial on AA administration in the immediate postnatal phase (19) and were selected if born within a predefined time span during that study and if they met current inclusion criteria. The study was designed as a randomized open trial and was performed in the NICU of the Erasmus MC – Sophia Children's Hospital, Rotterdam, the Netherlands. The study was investigator initiated with no funding from industry. The protocol was approved by the Erasmus MC Medical Ethical Review Board and written parental consent was obtained prior to study.

Experimental design

Within two hours after birth, infants were randomly assigned to receive during the first two postnatal days either glucose only (control group, n=7) or glucose and 2.4 g protein/(kg·d) as AAs (Primene 10%, Baxter, Clintec Benelux NV, Brussels, Belgium) (intervention group, n=8).

The administration of glucose solution or the AA and glucose solution was accomplished by continuous infusion. Lipids and/or (minimal) enteral feedings were withheld during the study period. None of the infants received exogenous albumin infusions during the study. The hospital's pharmacy dissolved L-[1-^{13}C]leucine (99% enriched, Cambridge Isotope Laboratories, Andover, MA, USA) in normal saline and tested it for sterility and pyrogenicity. We infused it (prime: 15 μmol/kg; continuous 15 μmol/(kg·h)) with the use of an infusion pump (Perfusor fm; B Braun Medical B.V., Oss, the Netherlands).

Arterial blood samples (0.4 mL) were drawn before the isotope infusion (baseline) and after 4 and 5 hours of infusion. The blood samples were immediately put on melting ice and centrifuged (2500 × *g*, 10 min, 4 °C), after which the plasma was stored at -80°C until analysis.

Analytic methods

To isolate plasma albumin (20), samples (50 μL plasma) were deproteinized and washed with 10% trichloroacetic acid. To the protein pellet, water and 1% trichloroacetic acid in 96% ethanol were added and the sample was centrifuged. The supernatant was mixed

with 26.8% ammonium sulfate to precipitate albumin overnight. The pellet was then dissolved in 0.3 mol NaOH/L and again precipitated with 2 mol perchloric acid/L. After washing, the new pellet was redissolved in 6 mol HCl/L and hydrolyzed for 24 hours, after which the acid was dried under nitrogen and dissolved in water. AAs were isolated using a cation-exchange column, derivatized with ethylchloroformate, and enrichment was measured on a gas chromatograph – combustion – isotope ratio mass spectrometer (GC-C-IRMS; Delta XP; Thermo Electron, Bremen, Germany) as previously described (21).

As albumin precursor we used the plasma [1-^{13}C]α-ketoisocaproate (α-KIC, the keto acid of leucine) enrichment at plateau which had already been measured as described in our preceding paper (16). While liver amino acyl-tRNA enrichment forms the true precursor, its use requires tissue biopsies and technically demanding assays. Nevertheless, α-KIC enrichment adequately represents leucyl-tRNA enrichment and is valuable in this type of research (22, 23). Plasma albumin concentrations were routinely measured on a Roche Hitachi 912 (Roche Diagnostics, Basel, Switzerland).

Calculations

The FSR reflects the fraction of the intravascular albumin pool that is renewed per unit of time (%/d) and can be calculated as follows:

$$FSR = \frac{E_{leu-alb,t_2} - E_{leu-alb,t_1}}{E_{KIC}} \times \frac{24}{t_2 - t_1} \times 100\%$$

where $E_{leu-alb}$ is the enrichment in mole percent excess (MPE) of incorporated leucine in albumin at t_2 and t_1 (at 5 and 4 hours after the start of infusion, respectively) and E_{KIC} is the mean enrichment in MPE of the precursor, i.e. plasma α-KIC, at both time points.

The ASR represents the absolute amount of albumin that is produced per unit of time (mg/(kg·d)), and can be calculated as follows:

$$ASR = FSR \times C_{alb} \times vol_{bl} \times (1 - Ht) \times weight^{-1}$$

where C_{alb} is the plasma albumin concentration in g/L, vol_{bl} is the infant's total blood volume in mL (assumed to be 75 mL/kg body weight (24, 25)), (1 – Ht) is the fraction of blood which is plasma, and weight is birth weight in kg.

We also calculated the contribution (%) of albumin ASR in relation to whole body protein synthesis in percentage based on previously measured leucine turnover data according to the following formula:

$$contribution = \frac{ASR \times 0.104}{NOLD \times 131.2 \times 24 \times 0.001} \times 100\%$$

where NOLD is the non oxidative leucine disposal representing whole body protein synthesis in μmol/(kg·h) and which was calculated in an earlier study by our group (16). Furthermore, 0.104 represents the fraction of leucine residues in albumin on a weight basis, 131.2 is the mole mass of leucine, and 24 and 0.001 convert to day and milligram, respectively.

Statistics

Calculations were made with Microsoft Office – Excel software (version 2000; Microsoft Corp, Redmond, WA, USA) and all statistical tests were done with GraphPad Prism software (version 4.0; GraphPad, San Diego, CA, USA). Differences between control and intervention groups were tested by Mann-Whitney tests unless otherwise stated. Values are expressed as median (25^{th} – 75^{th} percentile) or as mean (SD) and significance level was set at $p<0.05$.

Results

Fifteen premature infants were studied: seven in the control group and eight in the intervention group. All infants were mechanically ventilated. Birth weight, gestational age, SD score for weight (26), sex, disease scores (Apgar and CRIB (27)), and antenatal steroid use to improve lung maturation were not significantly different between the groups (Table I). The birth weights of one infant in the control group and two infants in the intervention group were below 2 SD when related to gestational age. Blood gas parameters, whole blood glucose concentrations, and non-protein calorie intakes (only glucose) on the second day of life did not differ between groups (Table II). Because the

Table I: Clinical characteristics[1] [2]

	Control	Intervention
No. (male/female)	7 (2/5)	8 (4/4)
Weight (kg)	0.960 (0.780 – 1.080)	0.940 (0.770 – 1.070)
Gestational age (wks)	26.7 (26.4 – 28.9)	26.9 (26.6 – 29.9)
SD-score for weight (26) (SD)	-0.53 (-0.75 – -0.11)	-1.14 (-2.22 – -0.19)
CRIB score (27)	5 (2 – 8)	3 (1.3 – 5)
5 min Apgar score	9 (7 – 9)	9 (8 – 10)
Antenatal corticosteroids (no/yes)	(2/5)	(1/7)

[1] Values expressed as median (25th – 75th percentile).

[2] There were no statistical differences between groups (Mann-Whitney).

Table II: Study parameters on the second day of life[1]

	Control (n=7)	Intervention (n=8)
Non-protein energy intake (kcal/(kg·d))	33.3 (30.7 – 36.9)	31.2 (26.0 – 32.9)
AA intake (g/(kg·d))[2]	0	2.3 (2.3 – 2.4)
Nitrogen balance (mg N/(kg·d))[2]	-110 (-133 – -56)	156 (116 – 226)
Blood urea nitrogen (mmol/L) [2]	6.2 (5.8 – 6.9)	9.7 (7.6 – 11.8)
Glucose concentration (mmol/L)	4.9 (3.1 – 6.2)	3.9 (3.0 – 4.8)
pH	7.31 (7.25 – 7.33)	7.31 (7.28 – 7.38)
Base excess (mmol/L)	-5 (-6 – -4)	-6 (-6 – -3)

[1] Values expressed as median (25th – 75th percentile).

[2] Statistically significant, $p<0.05$ (Mann-Whitney).

intervention group received AAs, blood urea nitrogen concentration and the nitrogen balance were higher in these infants.

The mean leucine enrichments in albumin in the control group were 0.243 (0.12) and 0.289 (0.13) mole percent excess (MPE) after 4 and 5 hours of infusion, respectively. In the intervention group, enrichments were 0.201 (0.050) and 0.249 (0.050) MPE. The mean α-KIC enrichments at plateau were 7.16 (0.56) MPE in the control group and 5.18 (0.46) MPE in the intervention group.

The intervention group showed significantly higher albumin FSR than the control group (Figure 1). Albumin plasma concentration was measured in 5 out of 7 infants in the control group and in 6 out of 8 infants in the intervention group; it was significantly

Figure 1. Albumin FSR in %/d in the control (n=7) and intervention (n=8) groups. Presented as median and interquartile range. The intervention group had a significant higher albumin FSR (p=0.029; Mann-Whitney).

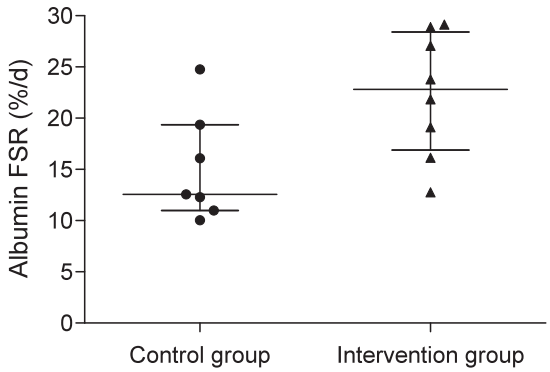
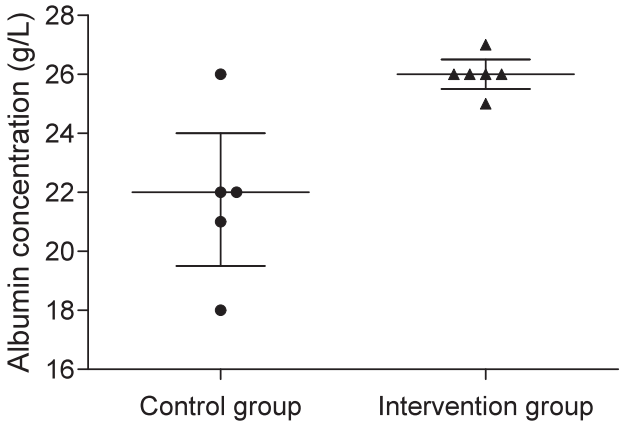


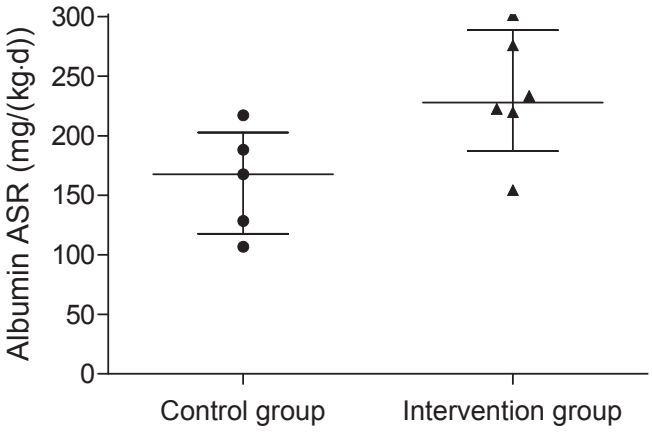
Figure 2. **Albumin plasma concentration in g/L in the control (n=5) and intervention (n=6) groups. Presented as median and interquartile range. The intervention group had a significant higher plasma albumin concentration (p=0.030; Mann-Whitney).**



higher in the intervention group (Figure 2). The calculated ASR was also higher in the intervention group (Figure 3).

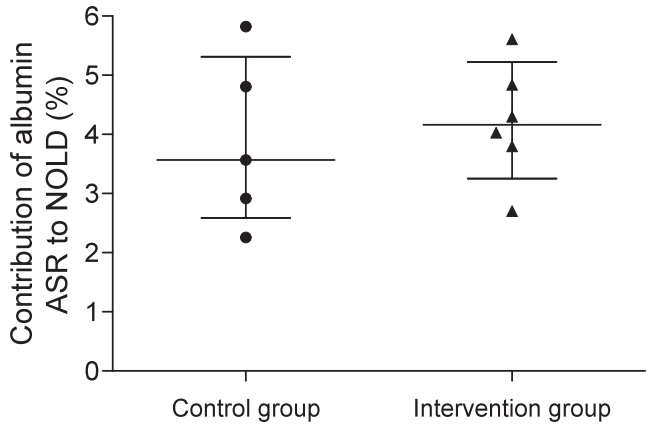
Because we had also obtained leucine turnover data (16), we were able to compare the albumin ASR with the whole body protein synthesis rate. The median NOLD (a measure of protein synthesis) increased from 130 (122 – 172) to 185 (169 – 203) μmol leucine/(kg·h) upon AA administration (p=0.030). The proportion of leucine used for

Figure 3. **Albumin ASR in mg/(kg·d) in the control (n=5) and intervention (n=6) groups. Presented as median and interquartile range. The intervention group had a significant higher albumin ASR (p=0.030; Mann-Whitney).**



albumin synthesis relative to whole-body NOLD was approximately 4% in both subject groups (Figure 4), which implies that AA administration stimulates albumin synthesis and whole-body protein synthesis at a similar rate.

Figure 4. Contribution in % of albumin synthesis (ASR) to whole body protein synthesis (NOLD) in the control (n=5) and intervention (n=8) groups. Presented as median and interquartile range. There was no difference between groups (Mann-Whitney).



Discussion

Our data are consistent with the assumption that parenteral AA administration in premature neonates stimulates albumin synthesis. For on the second day of life albumin FSR, ASR, and plasma albumin concentrations were significantly higher in premature infants who had received parenteral AAs from birth onwards.

Plasma albumin concentration is governed by four processes; changes in synthesis, degradation, intravascular space, and transcapillary escape. In the present study, we were not able to measure the three latter processes, but we speculate that increased synthesis is the primary cause for the higher plasma concentration noted.

Alternatively, a rise in albumin synthesis does not automatically coincide with a parallel rise in concentration. Albumin is a negative acute-phase protein, which means that its concentration will fall during an inflammatory event. Such falls have been described during cholecystitis (28), hemodialysis (29), cancer (30), and in head trauma patients (31), despite a coinciding rise in albumin FSR. Cytokines might be responsible for this paradoxical increase (29, 32). The lowered concentrations probably result from concomitant increases in catabolic rate and transcapillary escape.

The albumin FSR in healthy adults is about 6-8 %/d (8, 9, 32-36) and seems unresponsive to intravenous nutrients (37). Meals, however, will increase albumin synthesis (7, 8, 38). A recent study showed that the protein portion of meals is the effective component responsible for this increase (9). Adults suffering from chronic hemodialysis were also found sensitive to nutrition, as albumin FSR improved after intradialytic administered nutrition (20). Overall, it seems that albumin synthesis in adults is more responsive to gastro-intestinal nutritional uptake than intravenous nutrition, as was also demonstrated after surgery (39) and in rats (40). Our findings in human neonates and findings in young piglets suggest that other metabolic mechanisms might be regulating albumin synthesis in younger individuals, whose albumin synthesis is also responsive to parenteral nutrition (41, 42).

Consistent with the general finding that younger individuals have higher metabolic rates than adults, higher albumin FSR, ranging from 15 to 20 %/d, have been found in 12-month-old infants (43, 44). Bunt et al found values of 14 %/d in fasted premature infants with a gestational age of 28 weeks on the first postnatal day (21). Yudkoff et al calculated a mean albumin FSR of 12%/d in parenterally fed, premature neonates with appropriate size-for-gestational-age (which was 28 weeks), after approximately 1 week of life (45). These figures correspond well with the synthesis rates we observed in this study. Yet, unlike Bunt et al (21), we did not find clear correlations between the FSR and SD-scores for weight related to gestational age. This might have been the result of reduced power in our study or interference by our nutritional intervention.

We calculated that albumin constitutes about 4% of all proteins synthesized in the body. Besides, in healthy humans and rats, it was estimated that of all proteins synthesized in the liver, including those not excreted but produced for intrahepatic maintenance, 15% was albumin (34, 38). Combining these figures reveals that the liver would contribute over 25% to whole body protein synthesis. Normal hepatic functioning would therefore seem to be of vital importance.

Apart from all the important roles of albumin mentioned in the introduction, increasing the albumin FSR is also interesting from a nutritional point of view. A higher albumin ASR makes premature infants less vulnerable to catabolic insults through the temporary storage of AAs in albumin, preventing excess AAs from being oxidized. Later, during low protein intake or increased demands, body protein stores can be spared albeit at the cost of albumin breakdown, thereby releasing free AAs.

Especially in the first 24 hours after premature birth, nonprotein energy intake is usually very low (~30-35 kcal/(kg·d)) and less than desirable. Recently, energy expenditure was measured in premature infants during the first few days after life having comparable caloric intakes (46). Energy expenditure was estimated at 29-35 kcal/(kg·d), thus leaving, at an intake of approximately 30 kcal/(kg·d), no calories for net energy storage

or growth. As a consequence, AA efficacy in terms of anabolism is usually moderate in that a large fraction will be irreversibly oxidized (16).

Carbohydrate intake is limited due to potential hyperglycemia and fluid restrictions. Parenteral lipids are also often withheld in the first 24 hours after premature birth, as neonatologists fear pulmonary disease, hypertriacylglycerolemia, and high free fatty acid concentrations leading to competition with bilirubin binding on albumin (47).

Albumin is the main transport vehicle for fatty acids to and from the tissues according to metabolic demands. Notwithstanding the fact that lipids in blood are largely in the form of triacylglycerols, the turnover and utilization of fatty acids bound to albumin is high, thus making fatty acids an important contributor to lipid metabolism (48). Providing AAs directly after birth increases albumin synthesis and subsequent binding capacities, which theoretically improve the tolerance of intravenous lipids. Apart from the advantage of delivering essential fatty acids, the high caloric content makes immediate commencement of lipids after birth beneficial by improving the energy balance. This in turn might stimulate protein synthesis even more.

A recent study with high dose AAs and lipids initiated immediately after birth, demonstrated high anabolic use of AAs, probably due to a higher energy availability (49). We would like to speculate that an increased albumin synthesis rate was at least partially responsible for the increased tolerance of lipids. More clinical trials are required to determine efficacy and safety of starting parenteral administration of lipids together with high dose AAs immediately after birth to premature infants.

A potential limitation of this study is that the hydrolyzed protein pellet may not have contained pure albumin. Jacobs et al earlier reported that after simple ethanol extraction about 8% of proteins were contaminants (50). By adding ammonium sulfate we aimed to eliminate some of the contaminating proteins (20). However, even if purification of the protein pellet was still incomplete, the vast majority must have been albumin.

In conclusion, we have shown that introducing AAs immediately after birth to premature neonates stimulates not only whole body protein synthesis, but also albumin synthesis. This finding might have important implications in view of the vital roles of albumin, among which serving as an antioxidant and binding bilirubin and free fatty acids. Improving albumin synthesis might, therefore, have major impact on later outcome.

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5 Long term safety and efficacy aspects of early amino acid administration in preterm infants

Frans WJ te Braake, Chris HP van den Akker, Nynke Weisglas-Kuperus, and Johannes B van Goudoever

Submitted

Introduction

Since the early 1970s the availability of parenteral nutrition has enabled the administration of lipids and amino acids (AAs) to preterm infants in the neonatal phase. However, administration of first generation AA solutions resulted in serious metabolic disturbances, impeding their use in preterm infants, and impacting on nutritional regimens up till the present day.

A large number of studies has been conducted since, on what is described as *aggressive* early AA administration. In 2005 in this journal, we published data of a randomized clinical trial, in which we investigated safety and efficacy aspects of early AA administration in a group of 135 very low birth weight (VLBW) infants (1). Our results reconfirmed metabolic safety of early AA administration while a catabolic state was converted into anabolism.

Although it is known that postnatal growth restriction may have long-lasting adverse effects, such as short stature and compromised neurodevelopmental outcome (2, 3), evidence for improved long-term outcome as a result of early AA administration is limited (3). In fact, recently some concerns have been raised about safety and efficacy of early aggressive AA administration (4, 5).

In this short report, we describe neurodevelopmental outcome at two years of age in our previous cohort of 135 very low birth weight infants. We hypothesized that early AA administration at a dose of 2.4 g/(kg·d) does not negatively affect neurologic development.

Methods

In our initial study we included 66 infants with a birth weight below 1500 g who received AAs (2.4 g/(kg·d)) directly from birth onwards (intervention group), and 69 infants who received glucose only for the first two days of life (control group) (1). Of the 135 infants,132 had a gestational age less than 32 weeks and were therefore considered eligible for neurodevelopmental follow-up and anthropometric assessment at 2 years of corrected age. All parents gave written permission to use the follow-up data for statistical analyses.

The prevalence of handicaps, such as cerebral palsy, visual and hearing impairments was documented. The Mental Developmental Index (MDI) was assessed by the Bayley Scales of Infant Development (BSID), 2nd edition. Furthermore, anthropometric data were recorded.

Data were analyzed using the statistical package SPSS version 15.0 (SPSS Inc, Chicago, USA). Data are described as mean ± SD. A p-value <0.05 was considered statistically significant.

Results

In the initial study we included 132 infants with a gestational age less than 32 weeks. Eighteen infants died in the neonatal phase (ten infants in the control group; eight infants in the intervention group). Twelve of the 114 surviving children were lost to follow-up (eight in the intervention and four in the control group). Consequently, in 102 children (89%, 47 in the intervention and 55 in the control group), follow up data were available. There were no differences in demographic data except for a trend towards more males in the intervention and more females in the control group (Fisher's Exact Test p=0.05).

Table I shows neurologic outcome at two years of age. No differences were observed between the intervention and control group as a whole. In boys but not in girls, the

Table I: Neurodevelopmental outcome at two years of age in the intervention and control groups.

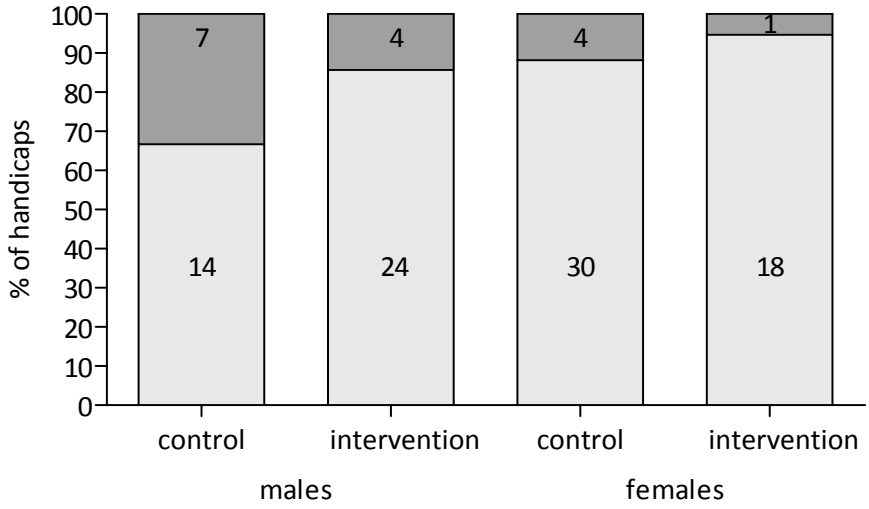
		Intervention (n=47)	Control (n=55)	Total (n=102)
Any handicap *	Absent	42 (89%)	44 (80%)	86
	Present	5 (11%)	11 (20%)	16
Neurology	Normal	38 (81%)	47 (86%)	85
-	Mildly abnormal	8 (17%)	4 (7%)	12
-	Cerebral palsy	1 (2%)	4 (7%)	5
Visual deficiency	Absent	47 (100%)	53 (96%)	100
-	Present	0 (0%)	2 (4%)	2
Hearing deficiency	Absent	46 (98%)	54 (98%)	100
	Present	1 (2%)	1 (2%)	2
Mental Developmental Index	mdi >85	34 (76%)	36 (77%)	70
(BSDI II)**	mdi 85-70	8 (18%)	6 (13%)	14
	mdi <70	3 (7%)	5 (11%)	8

* visual/hearing deficiency, cerebral palsy or MDI<70

** visual/hearing deficiency and cerebral palsy (n=8) and refusals (n=2) excluded

Figure 1: percentage of handicaps* in males and females in the intervention versus the control group. Light gray represents the absence and dark gray represents the presence of handicaps.

***visual/hearing deficiency, cerebral palsy and/or MDI<70**



percentage of handicaps (e.g. visual/hearing deficiency, cerebral palsy and/or MDI<70) in the intervention versus the control group tended to be lower (p=0,10) (Figure 1). Table II shows anthropometric data of the included children. Again, no differences were observed between groups.

Table II: Weight and head circumference at two years of age in the intervention and control groups.

	Intervention (n=44)	Control (n=50)
Weight (kg)	11.6 ± 1.83	11.4 ± 1.68
Head circumference (cm)	48.5 ± 1.85	48.2 ± 1.74
ΔSD weight 2 y minus birth (SD)	0.122 ± 3.10	-0.112 ± 1.39
ΔSD head circumference 2 y minus birth (SD)	-0.04 ± 1.58	-0.30 ± 1.52

Discussion

In the past decade, a large number of studies have been published on early AA administration and its beneficial effects in terms of promoting anabolism in the early postnatal phase. Recently we demonstrated additional benefits upon early AA administration in terms of increased albumin and glutathione synthesis rates (6, 7). In our study,

however, there was no benefit in terms of growth at two years of age. In most studies anthropometric measurements improve at discharge after early AA administration whereas this effect is disappearing at 18 months of age (8-11). Whether rapid postnatal weight gain has a favorable effect on neurodevelopment is yet unclear. Still, favorable effects of a rapid postnatal weight-gain on neurodevelopment, as assessed by BSID, were found in a study of Latal-Hajnal et al. in 219 VLBW infants (12). No differences, however, were found in a study including over 1000 extremely low birth weight infants, conducted by Poindexter et al. at 18 months corrected age (8).

In this exploratory study we demonstrate that providing 2.4 g AAs/(kg·d) seems safe with respect to the handicap rate at two years of age. On the other hand, no apparent clinical benefits were demonstrated as yet although there are multiple theoretical advantages of being anabolic during the early postnatal phase.

Neurodevelopmental outcome in preterm infants is dependent on multiple variables, of which one is nutrition. We therefore do not suggest that deprivation of two days of AAs will have a major clinically measurable effect on long-term outcome.

While girls tended to have a more favorable neurodevelopmental outcome than boys, the handicap rate in boys in the intervention group tended to be lower in the control than in the intervention group. The number of studied children is relatively small, however and the age of 24 months is too young for definitive conclusions regarding more neurocognitive outcome at a later age.

In conclusion, in this exploratory study, no negative effects of early AA administration on postnatal growth and handicap rate were found. In our opinion, this should strongly encourage a nutritional strategy including early AA administration, seeing its beneficial effects in the early neonatal phase. Long term follow-up is mandatory to define whether early aggressive AA administration will have a a, perhaps subtle, effect on long-term, neurocognitive outcome.

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6 Novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS)

Henk Schierbeek, Frans WJ te Braake, Jean-Philippe Godin, Laurent-Bernard Fay, and Johannes B van Goudoever

Rapid Commun Mass Spectrom. 2007;21(17):2805-12

Abstract

A novel analytical method using liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS) was developed for measuring glutathione (GSH) fractional synthesis rate (FSR) in neonates after infusion of [1-^{13}C]glycine as a tracer. After transformation of GSH into GSSG, its dimeric form, the intra erythrocytic concentration and ^{13}C- isotopic enrichment of GSH were determined using 200 µL of blood. The results showed that using LC-IRMS, the concentration (range of µmol/mL) was reliably measured using norvaline as internal standard with precision better than 0.1 µmol/mL. In addition, the ^{13}C-isotopic enrichment measured in the same run gave reliable values with excellent precision (with sd lower than 0.3 ‰) and accuracy (measured between 0 and 2 APE). The inter assay repeatability of δ^{13}C of norvaline used as internal standard with *in vivo* samples was assessed at -26.07 ± 0.28 ‰ with CV at 1.1%. The FSR calculated either with GSH or GSSG showed similar results with slightly higher values for GSSG (41.6 ± 4.7 and 46.5 ± 4.4 respectively). The slighly lower FSR of GSH is probably due to interfering compounds in the biological matrix. Successfully used in a clinical study, this rapid and reliable method opens up a variety of kinetic studies with relatively low administration of tracer infusates, reducing the total cost of the study design. Besides, the small volume of blood needed enables studies even in extremely small subjects, such as premature infants as reported in this study.

Introduction

Glutathione (GSH), or L-γ-glutamyl-L-cysteinyl-glycine, is a tripeptide consisting of glutamate, cysteine and glycine. It is synthesized *de novo* in nearly all mammalian cells. Glutathione is present in its monomeric, reduced form (GSH), and its dimeric, oxidized form (GSSG). GSH can be oxidized non-enzymatically *in vivo* to GSSG by electrophilic substances, such as reactive oxygen species. GSH is regenerated from GSSG by the enzyme glutathione reductase. Glutathione is synthesized de novo in two steps: first, catalyzed by glutamylcysteine synthetase, a glutamate is covalently bound to a cysteine residue – the rate-limiting step – and second, catalyzed by glutathione synthetase, glycine is added (1, 2). Nearly all mammalian cells produce glutathione in large amounts, reaching intracellular concentrations within the millimolar range (3). In plasma, it is mainly present in its oxidized form such as GSSG or protein GSH disulfite, at levels in the micromolar range (2-20 µmol/L). The monomeric form, GSH, is actively involved in many cellular functions, both as a cofactor for several enzymes and independently (4), it acts as antioxidant by scavenging radicals or xenobiotics (such as acetaminophen), plays a role in the synthesis of leukotrienes and prostaglandins, in cell proliferations, and is involved in many diseases, such as cancer.

It is quite common to measure GSSG concentration in addition to GSH in biological samples, as the GSH/GSSG ratio is considered to be inversely related to oxidative stress (5). To this aim, high performance liquid chromatography methods using electrochemical (6) or fluorometric detection (7) or nuclear magnetic resonance have been reported (8). However, concentration of GSSG in erythrocytes is very low and possible artifacts in sample handling might produce misleading results (9).

Glutathione metabolism has been studied in a variety of species and experimental settings (10-14). The emphasis, though, was largely on concentration measurements, with a minor role for measuring kinetics using stable isotope tracers. GSH has a rapid turnover, as reflected by its high fractional synthesis rate (FSR). In healthy adult volunteers, FSR is approximately 65-83 %/d (14, 15), which implies that all GSH is completely renewed in 36 hours. Therefore, static parameters such as concentration may not necessarily provide useful information if kinetic data are absent. Quantifying utilization and synthesis rates of GSH provides a dynamic insight into its metabolism under pathological conditions such as oxidative stress or in response to interventions. Nowadays, gas chromatography-mass spectrometry (GC-MS) techniques are used to study FSR with stable isotope tracers (^{13}C or ^{2}H incorporated into glycine or cysteine respectively) and a primed continuous infusion for 6 to 8 hours (16). In addition, FSR determination in erythrocytes requires suitable methods to measure both low level of isotopic enrichment in GSH and in its precursor. Typically, this information is obtained with the incorporation rate of tracer into GSH, using erythrocyte-free glycine or cysteine

as the precursor pool from which erythrocytes synthesize GSH (11). Studies in neonates are limited by the small amounts of blood that can be sampled. Then, only small amounts of stable isotope tracer can be used, lest metabolism is disturbed.

Although gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is now the state of the art to measure low isotopic enrichment in tracer studies, the targeted compound needs to be volatile after derivatization (17). Thus, for isotopic analyses of a tripeptide such as glutathione, the GC introduction might be quite laborious. Then, liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS) needs to be envisaged as well. The LC-IRMS device became commercially available in 2004 (18) after several other experiments to link LC to IRMS over the last decade (19-23). This coupling is based on a wet chemical oxidation process and presents few analytical constraints interfering with LC method development. Typically the LC flow rate must be lower than 600 µL/min, organic buffers are prohibited and the presence of salt and low pH facilitates CO_2 extraction. Various LC separations were developed using LC-IRMS for targeted compounds such as underivatized amino acids (18, 24, 25) and underivatised carbohydrates (26) but none specifically for routine isotopic analysis and concentration measurement in the frame of metabolic studies. Our aim here was to develop a novel and robust method to determine low GSH ^{13}C-isotopic enrichments requiring a low volume of blood, for use in e.g. premature infants. Therefore, a new method was designed based on stable isotope technique using liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS). To this purpose, we assessed GSH kinetics through the measurement of the ^{13}C-isotopic enrichment and the concentration of GSSG entity chemically produced by oxidation of GSH.

Experimental Section

Chemicals and reagents

GSH, glycine, norvaline, norleucine 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 and sodium hydroxide were purchased from Merck (Darmstadt, Germany). The [1-^{13}C]-labelled glycine was purchased from Cambridge Isotope Laboratories (Buchem, Netherlands). [1,2-^{13}C, ^{15}N]GSH was a gift from Cambridge Isotope Laboratory, CIL (Andover, MA, USA). Sterile water was purchased from Baxter BV (Utrecht, Netherlands) dithiothreitol (DTT) and N-ethylmaleimide (NEM) were purchased from Sigma (St Louis, USA).

Instruments

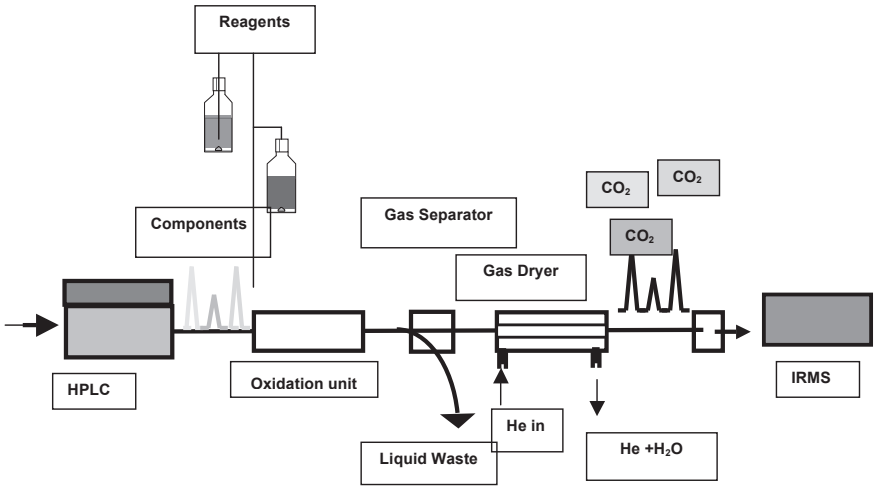
Experiments were carried out on a Delta XP (Thermo Electron, Bremen, Germany). The IRMS was operated at an accelerating voltage of 5 kV. The ion source was held at a pressure of 3.0 x 10^{-6} Torr, and ions generated by electron impact at 70 eV. Three faraday cup detectors monitored simultaneously and continuously the $CO_2^{+\cdot}$ 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 (Linde, quality 5.3, Hoekloos, Schiedam, Netherlands) was calibrated with known reference gasses (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 20 V measured on the first faraday cup (resistor 300 MΩ).

An LC-Isolink® interface (Thermo Electron, Bremen, Germany) was coupled to the Delta XP with slight modifications. The mixing "Tee" was replaced by a "Tee" with a smaller dead volume (Bester BV, Amstelveen, Netherlands) and the stainless steel connections for the reagents and acid were replaced by "No-Ox" material (1/8'' x 1.5 mm). A degasser (Alltech, Breda, Netherlands) was placed between the solvent bottles and the pumps to remove traces of air in the eluents. "No-Ox" material was used to prevent re-gassing of solvents. The temperature of the interface reactor was set at 99.9 °C. PEEK® tubing was used for connections between the auto sampler the LC column and the interface. In addition, the reagent bottles were degassed with helium during analysis. To avoid crystallization 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 before the mixing chamber of the interface to avoid any blockage of particles in the system.

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). Figure 1 shows a schematic view of the system used for the analysis of glutathione.

The isotopic enrichment of ^{13}C-glycine in human erythrocytes was measured on the same Thermo Finnigan Delta-XP isotope ratio MS coupled online with a trace gas chromatograph (Thermo Electron, Bremen, Germany) and a combustion interface type III (Thermo Finnigan, Bremen, Germany) equipped with a CTC PAL auto sampler (CTC, Zwingen, Switzerland). After separation using a capillary GC column VF1701, 30 m x 0,25 mm ID and 0,5 µm film thickness (Varian B.V. Middelburg, Netherlands), derivatised amino acids were combusted online at 940°C and introduced as CO_2 into IRMS ion source, where the C^{13}/C^{12} ratio was measured.

Figure 1. **Schematic overview of LC-IR Mass Spectrometer device.**



Isotopic calibration and isotopic measurement

CO_2 reference gas was introduced at regular intervals at the beginning of each run for 20 s at a level of 3.0 ± 0.2 V on cup one (resistor 300 MΩ) and used for calibration.

The $^{13}C/^{12}C$ abundance ratio was expressed as δ ^{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:

$$\left[\frac{100 \times R \times ((\delta^{13}\ C/1000)+1)}{1+R \times ((\delta^{13}\ C/1000)+1)}\right]$$

Atom % =, 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:

$$\text{MPE} = \left[\frac{APE}{100+APE} x100\right]$$

Clinical study design

The study design was approved by the Erasmus MC Medical Ethical Review Board Committee and informed parental consent was obtained prior to the study. The study

population consisted of very low birth weight infants (birth weight <1500 g) admitted to the neonatal intensive care unit. A primed (40 µmol/kg) continuous infusion of 1-^{13}C labelled glycine (20 µmol/(kg·h)) was administered intravenously for 6 hours. Blood samples were taken after respectively 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.

Glutathione determination

Before analysis erythrocytes were disrupted by freezing and thawing (27). Then 40 µL of a mixture of 1.53 µmol/mL norleucine and 8.54 µmol/mL norvaline was added as internal standard and the samples were deproteinized by adding 200 µl of 6 % g/v perchloric acid, left for 10 min on ice and finally centrifuged at 10'000 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 of perchloric acid was precipitated and removed by centrifugation at 10'000 G for 10 min. 100 µL of 0.5M $NaHPO_4$ was added to maintain pH 9. The supernatant was filtered through 0.2 µm Nylon membrane filters (Nylon, Alltech, Breda, Netherlands). Then, 150 µL was transferred in a sample vial and 20 µL was injected for analysis of both GSH concentration and ^{13}C- isotopic enrichment by LC-IRMS. The remaining supernatant was used for analysis of both concentration and ^{13}C- isotopic enrichment of glycine by GC-C-IRMS.

Glycine determination

Aliquots of 200 µl of the remaining supernant were poured on a 1 mL AG50 W-X8, H^+ cat-ion exchange column (Biorad, Richmond, Virginia, USA). Columns were washed thoroughly with 5 mL water to remove non amino acid contaminants. Amino acids were eluted with 3 mL 6 M NH_4OH and dried under nitrogen at 50°C. Then, the dried residue containing glycine among other amino acids was converted to its *N*-ethoxycarbonylethyl ester derivative (28, 29). Finally aliquots of 1 µL of the chloroform suspension containing amino acid derivatives were introduced into the GC-C-IRMS system described previously.

Analytical LC-IRMS conditions

Samples were introduced using a Midas auto sampler (Spark) and analysed with a linear high-pressure gradient (as reported in Figure 1). Glutathione analyses (concen-

tration and isotopic enrichment) were performed on a Sielc primesep 100 mixed mode column (250 × 3.2 mm, 5 µm), (Aurora Borealis, Schoonebeek, Netherlands) at room temperature, (24 ± 2) °C. The LC flow rate was 500 µL/min. The LC gradient was linearly increased from 4% to 40 % 1 molar H_3PO_4 (pH 2.2) in 15 min, followed by a linear increase to 80 % 1 molar H_3PO_4 (pH 2.2) in 5 min and held for 8 min. Sodium peroxodisulfate 0.84 mol/L in sterile water was used 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 was 30 µL/min each.

Calculations and data evaluation

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 fractional synthesis rate (FSR) of glutathione and was calculated according to the equation described below (equation 1):

$$FSR = \frac{\text{slope}\, E_{[1-^{13}C]GSSG_{t4,5,6}}}{E_{[1-^{13}C]glycine}} \times 24h \times 100\%$$

^{13}C isotopic enrichment of GSSG was measured by LC-IRMS. 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-erythrocitic glycine, the precursor, in MPE at steady state.

Results & Discussion

In this study, a novel LC-IRMS method was developed to separate underivatised amino acids, underivatised GSH and GSSG with a phosphoric acid gradient condition in 2000 sec (33 min) as illustrated in Figure 1. In order to obtain better chromatographic resolution between GSH and amino acids eluted near GSH peak (Pro and Cys), helium flow in the separation unit was increased (from 1 to 2 mL/min) to reduce peak width (from 85 sec to 60 sec). This modification affected the overall sensitivity of the analyses without compromising GSH isotopic and concentration analyses in blood erythrocytes.

Transformation of GSH into GSSG and its separation in LC-IRMS

Reducing agents such as dithiothreitol (DTT) or N-ethylmaleimide (NEM) are normally used to reduce GSSG entity present in low amounts in blood or to prevent oxidation

immediately after sampling, respectively. However, these reagents evoked disturbing peaks when analyzing them with LC-IRMS (very abundant peaks at the beginning of chromatogram causing elevated baseline background). Moreover, GSH is very susceptible to oxidation, which necessitates strict sample collection and storage protocols. A recent paper indeed reported the artifactual oxidation of GSH after sample treatment with different deproteinizing agents and subsequent alkalinisation (30). We found that GSH is completely oxidized as early as after 4 hours at room temperature at pH 8-9. In addition, the area where GSH elutes in the chromatogram was not totally free of other co-eluting compounds (as illustrated in Figure 2), which increases the risk of incorrect measurements (either concentration or isotopic enrichment). Therefore, to avoid the problematic baseline separation of GSH, all GSH was completely oxidized and measured as GSSG. The complete transformation of GSH into GSSG was performed in 4 hours and monitored with the disappearance of GSH peak by LC-IRMS (as illustrated in Figures 3a and 3b). GSSG was eluting in a part of the chromatogram free of other co-eluting compounds as shown in Figure 3b. ^{13}C-isotopic enrichment as well as concentration of the total GSH pool was then reliably measured in this condition.

Measurement of concentration of GSSG

The concentration of the tracee is an important parameter in any metabolic study. Therefore, two internal standards (I.S.) (norvaline and norleucine) were added and eluted closely to the GSH and GSSG peaks, respectively. This spiking of I.S. allowed

Figure 2. LC-IRMS chromatogram of a 0.25 mmol hydrolysate amino acid standard mixed with 0.5 mmol glutathione standard solution at low pH.

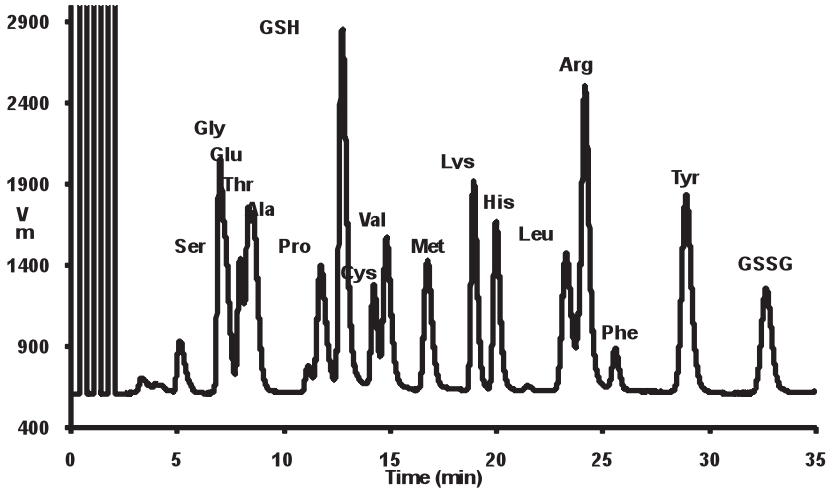


Figure 3a. **LC-IRMS chromatogram of glutathione analysis in erythrocytes of human blood at pH 4-5.**

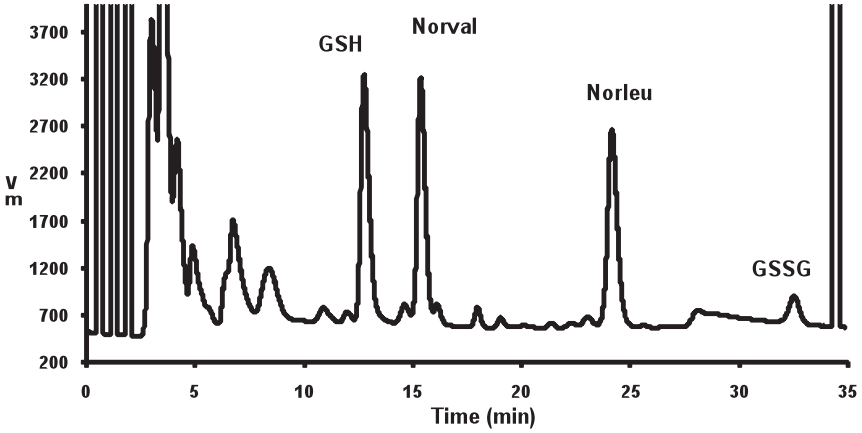
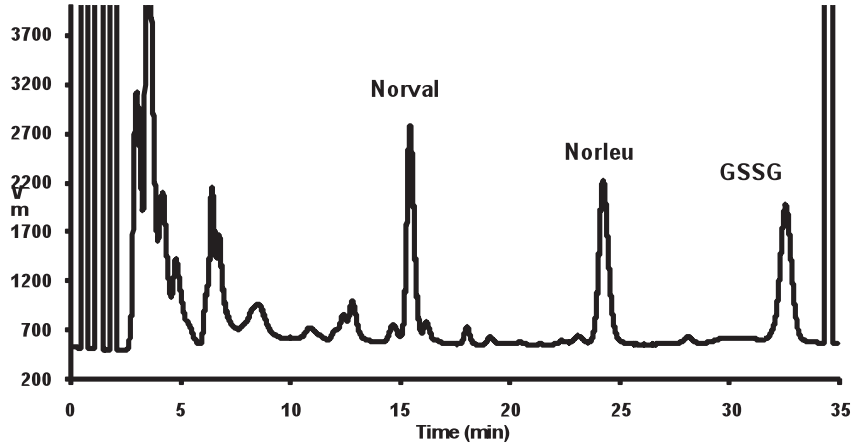
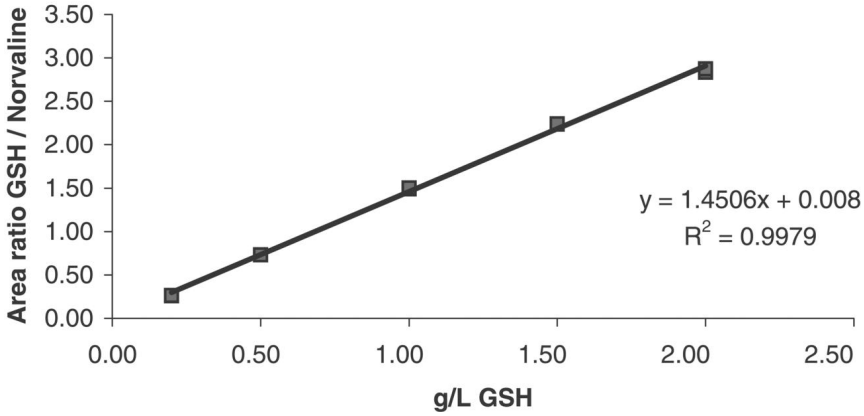


Figure 3b. **LC-IRMS chromatogram of glutathione analysis in erythrocytes of human blood as its oxidized form GSSG (pH 8-9).**



to obtain the GSH and GSSG ratios versus I.S. and thus to assess GSH and GSSG concentrations in blood. Five standard solutions of GSSG (using GSSG over I.S. area plotted against the µmol/mL of GSSG injected) were measured between 0.2 to 2 mg/mL. A linear relationship was obtained and gave y = 1.4506 x + 0.008. The regression coefficient (r^2) was calculated at 0.998 (Figure 4). The area of GSSG measured was between 17.7 ± 0.25 V.s to 191 ± 1.6 V.s. Table I shows GSSG concentrations in blood measured with only 200 µL of samples for four premature neonates. The concentration of GSSG measured through different days and showed good reproducibility (CV of 12.3 %, when measured as duplicates). The mean value (0.54 ± 0.07 µmol/mL)

Figure 4. **Calibration curve for measurement of glutathione concentration in human blood erythrocytes.**



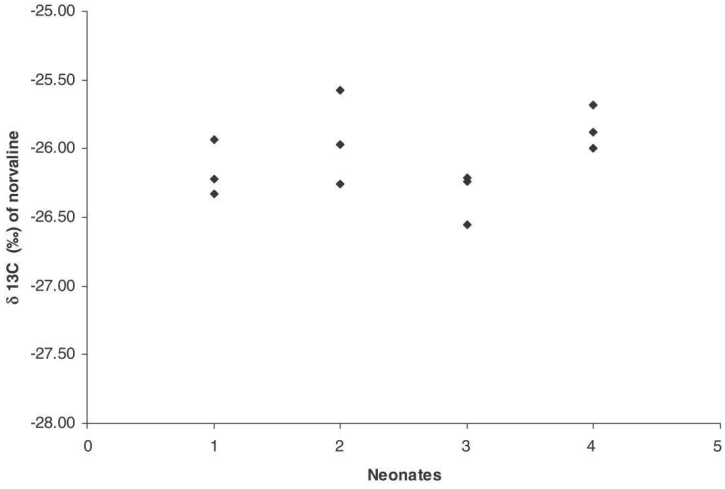
of GSSG equivalent to the concentration of GSH was consistent with values reported in the literature (31). These findings show that LC-IRMS can analyze concentration of metabolites in blood with good precision (better than 0.1 µmol/mL) and a detection range of 0.1-5 µmol/mL.

Table I. **Concentration and ^{13}C- isotopic enrichments of GSSG in 4 different neonates**

CV (%) = sd / mean x 100

	Time (hour)	Level (µmol/mL) of GSSG	sd (n=2)	δ ^{13}C (‰) of GSSG	sd (n=2)
neonate A	4H	0.55	0.06	5.48	0.68
	5H	0.58	0.001	8.54	0.55
	6H	0.61	0.004	13.51	0.64
neonate B	4H	0.61	0.02	-0.70	0.03
	5H	0.45	0.001	4.23	0.30
	6H	0.52	0.003	7.86	0.18
neonate C	4H	0.49	0.02	10.86	0.03
	5H	0.45	0.0003	15.61	0.44
	6H	0.44	0.003	22.81	0.04
neonate D	4H	0.53	0.02	-2.22	0.75
	5H	0.60	0.02	3.67	0.15
	6H	0.59	0.02	8.51	0.33
Mean		0.54			
sd		0.07			
CV (%)		12.3			

Figure 5. Variation of δ ^{13}C (‰) of norvaline added as internal standard and spiked in blood erythrocyte for 4 different neonates. Blood was collected at three time points and corresponding values are shown. Each point corresponds to duplicate injections.



Repeatability and accuracy of isotopic measurement

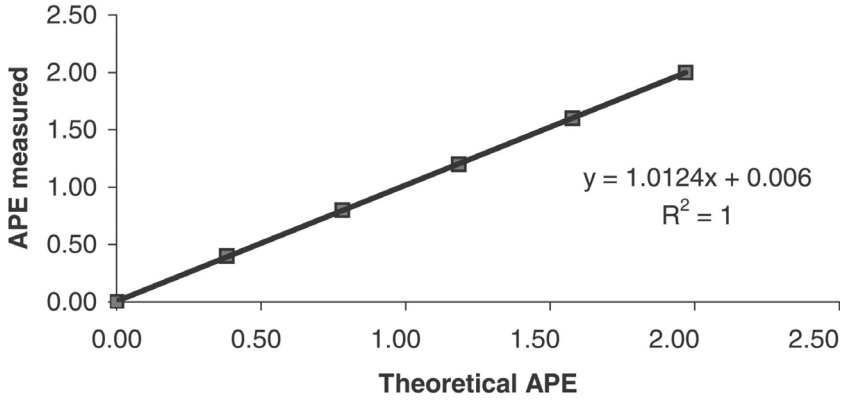
As illustrated in Figure 5, the intra-assay repeatability assessed with norvaline measured with three different blood samples from the same neonate showed a sd measured at -25.95 ± 0.20 ‰ and a CV calculated at 0.8 % (n=6). The inter-assay repeatability measured over different days was measured at -26.07 ± 0.35 ‰ (n = 24). The reproducibility (CV) was calculated at 1.3 %. For each neonate, the δ ^{13}C values of norvaline are very close. These values were in the same range as obtained with standard injection of I.S showing excellent isotopic precision as well as reproducibility of isotopic measurement at natural abundance.

Accuracy of isotopic measurement was assessed using standard calibration curve performed with various amounts of ^{13}C labelled GSH added to a fixed amount of natural GSH. By plotting measured APE versus theoretical APE between 0 and 2.5 APE (or δ ^{13}C values between -8.7 to 170.5 ‰), the curve was linear and a slope of 1.0124 was found (Figure 6). This shows that no isotopic fractionation occurred in the sample preparation and the analysis. The regression coefficient (r^2) was calculated at 1.000, showing excellent linearity.

Fractional synthesis rate (FSR) of glutathione

Blood samples were taken at regular intervals (4h, 5h and 6h) when erythrocyte [1-^{13}C] glycine levels were in steady state (according to results obtained by GC-C-IRMS and not shown). Figures 7a and 7b show a linear rise in time in both GSH APE and GSSG

Figure 6. **Calibration curve for measurement of [7-^{13}C]-glutathione enrichment in human blood erythrocytes using [1,2-^{13}C- ^{15}N] glutathione.**



APE. FSR of GSH and GSSG were calculated from these measurements using equation 1 reported before. Values for four patients are reported in Table II.

It follows that the glutathione pool of these patients was renewed approximately every two days. The calculated FSR slightly, but acceptably, differs for either of the forms of glutathione. In all cases the FSR for GSH is slightly lower than for GSSH. FSR of GSH was 41.6 ± 4.7 with a reproducibility (CV) calculated at 11.2 % and FSR of GSSG was 46.5 ± 4.4 with a reproducibility (CV) calculated at 9.6 % (n = 8). A possible explanation for the lower FSR for GSH is dilution of another co eluting (non amino

Figure 7a. **Graph of the increase of enriched GSH of a subject expressed in APE / hour.**

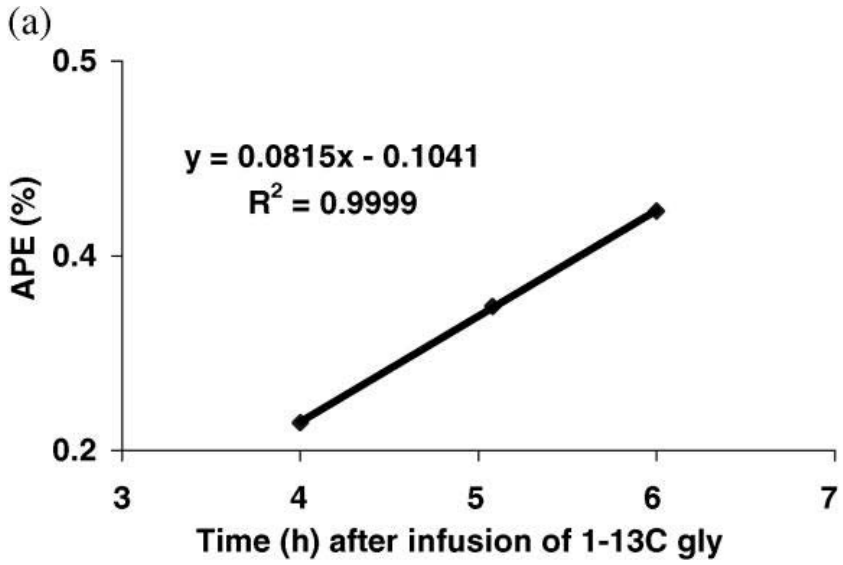
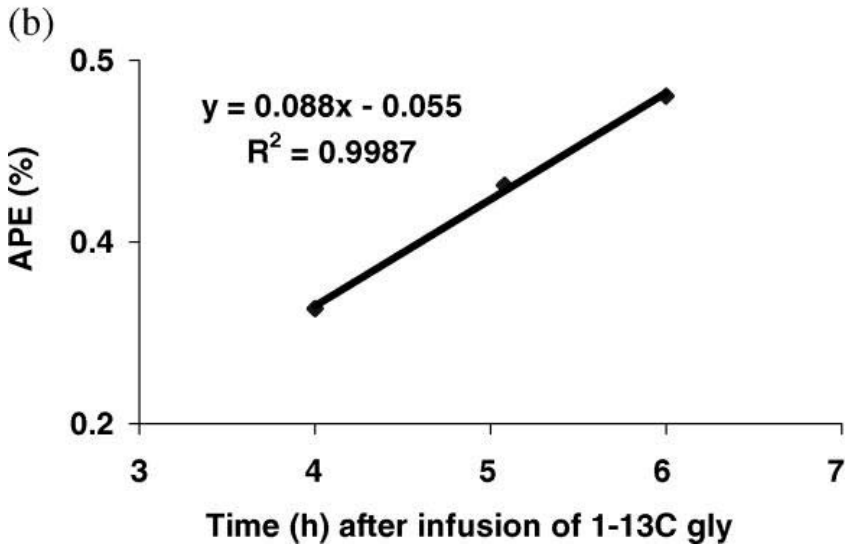


Figure 7b. **Graph of the increase of enriched GSSG of the same subject as shown in figure 7a expressed in APE / hour.**



acid) compound. For this reason, and also because the concentration is more precisely determined when measured as a single GSSG peak, we decided to measure GSSG, the oxidized form of GSH.

Table II. **Comparison of the fractional synthetis rates (FSR) of GSH and GSSG in four different subjects; GSH, GSSG and glycine analyses were carried out in duplicate.**

Sample	Compound	Precursor MPE In steady state	Slope APE %/ Hour	Slope MPE %/ Hour	FSR %/day
Neonate 1	GSH	3.90	0.0815	0.075358	46.36
	GSSG	3.90	0.0880	0.080882	49.77
neonate 2	GSH	2.93	0.0578	0.054642	44.76
	GSSG	2.93	0.0656	0.061562	50.43
neonate 3	GSH	3.66	0.0627	0.059001	38.70
	GSSG	3.66	0.0736	0.068554	44.97
neonate 4	GSH	3.53	0.057	0.053926	36.66
	GSSG	3.53	0.0641	0.060239	40.95
GSH	Mean	41.6	Sd	4.7	
GSSG	Mean	46.5	Sd	4.4	

Conclusion

This novel LC-IRMS method for measuring kinetics of glutathione using its oxidized form (GSSG) showed to be a powerful tool in metabolic studies in neonates. Only little pre-purification was necessary and the analyses reported here were fully automated. The measurements of both concentration and ^{13}C isotopic enrichment gave excellent results with only 200 µL of blood required, which is extremely important factor in neonatal studies. GSSG concentrations were found to be in agreement with results reported in literature. The precision and accuracy of isotopic enrichment at natural abundance and at higher isotopic enrichment gave excellent results showing no isotopic fractionation and an isotopic precision for GSSG in blood assessed at 0.3 ‰.

Acknowledgements

We would like to thank Katherine Belisle and Ron Trolard of Cambridge Isotope Laboratories for their generous gift of [1,2-^{13}C-^{15}N] glutathione.

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7 Glutathione synthesis rates and oxidative stress in extremely low birth weight infants in the first week of life

Frans WJ te Braake, Henk Schierbeek, Karien de Groof, Jan GM Huijmans, Mariangela Longini, Giuseppe Buonocore, and Johannes B van Goudoever

Submitted

Abstract

Synthesis of glutathione (GSH), the major intracellular antioxidant, might be impaired in preterm infants due to lack of substrate availability when introduction of amino acids is delayed. This might exacerbate oxidative stress. We hypothesized that preterm infants compensate for oxidative stress by upregulating GSH synthesis upon adequate nutritional support on postnatal day 6. We conducted an observational longitudinal stable isotope study in extremely low birth weight infants (birth weight <1000 g) to determine erythrocyte GSH concentrations and synthesis rates on postnatal day 2 (when receiving glucose only), and on postnatal day 6, (when receiving both parenteral and enteral nutrition). In plasma, we measured advanced oxidized protein products and dityrosine as markers of oxidative stress. Ten infants completed the study. Concentrations of advanced oxidized protein products and dityrosine increased significantly from day 2 towards day 6. In contrast, despite an overall increase of plasma precursor amino acids on day 6, GSH synthesis rates and concentrations were not different between day 2 and 6. In conclusion, these data indicate ongoing oxidative stress on postnatal day 6, which is not compensated for by an increased GSH synthesis rate, despite increased substrate availability.

Introduction

The transition from fetal to neonatal life coincides with many physiological changes. These are generally adequately anticipated for, e.g. by up- or downregulation of specific metabolites. Antioxidant production or its precursors are upregulated just prior to term birth in animals (1, 2) and humans (3), most likely to prevent excessive formation of so-called reactive oxygen species (ROS) such as free radicals, which evolve intracellularly upon exposure to the hyperoxic extrauterine environment relative to the intrauterine environment. Preterm infants, however, show high levels of protein and lipid (per)oxidation, whereas antioxidant concentrations remain low (4-6). Additional oxygen requirements in preterm infants add up to the formation of ROS evoking cellular damage, designated as oxidative stress. An association between oxidative stress and diseases such as bronchopulmonary dysplasia, retinopathy of prematurity, and periventricular leukomalacia has been established earlier (7-10).

Besides numerous metabolic functions, glutathione (GSH) is the body's main intracellular antioxidant. Its enzymatic synthesis depends primarily on the presence of its constituent amino acids (AAs): glutamate, cysteine, and glycine. Ironically, significant amounts of (parenteral) nutrition are often withheld in preterm infants during the direct postnatal period, because of feared intolerance and possibly because nutrition is considered less a priority directly after birth. Nevertheless, a delay in providing adequate nutrition, in particular AAs, might impede GSH synthesis and exacerbate oxidative stress. The more so, since cysteine and possibly glycine have been proposed to be essential for the preterm infant (11, 12). Indeed, several studies indicated failure in preterm infants to increase GSH concentrations in the first week of life when nutritional support was inadequate (4, 13). Low concentrations might be due to compromised synthesis because of lack of substrates, increased utilization as a result of oxidative stress, or a combination of both.

We hypothesized that preterm infants experience oxidative stress in the first days of life which is compensated for by an increased GSH synthesis rate upon full nutritional support on postnatal day 6. To test this hypothesis, we quantified markers of oxidative stress and GSH synthesis rates in preterm infants while receiving glucose only (postnatal day 2) and, subsequently, while receiving full nutritional support (postnatal day 6), provided both parenterally and enterally.

Methods

Study and Subjects

The study was designed as an observational study performed in the neonatal intensive care unit of the Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands. The protocol was approved by the Erasmus MC Medical Ethical Committee and informed written parental consent was obtained prior to study. This study took place prior to a change in nutritional policy which now includes AA administration starting directly following birth.

Subjects were extremely low birth weight (ELBW) infants (birth weight <1000 g). Each infant was administered parenterally stable isotopes for six hours on two separate occasions: on postnatal day 2 (study d2), and postnatal day 6 (study d6). Until completion of study d2, infants received glucose only. Afterwards, AAs (Primene 10%, Baxter, Clintec Benelux NV, Brussels, Belgium) and lipids (Intralipid 20%, Fresenius Kabi BV, 's Hertogenbosch, The Netherlands) were administered as separate solutions. Upon progression of enteral feeding, parenteral glucose intake was decreased. After the third day of life, all nutrient intakes, including enteral feedings were left to the discretion of the attending neonatologist. We excluded infants that received transfusions of erythrocytes during or within 12 hours prior to the study, or had known congenital abnormalities, chromosome defects, and metabolic, endocrine, renal, or hepatic disorders.

Study Endpoints

Primary endpoint of the study was antioxidant defense in response to increased nutritional intakes, as reflected by GSH synthesis rates in the first week of life and presence of oxidative stress. Secondary endpoint was capacity for GSH synthesis in early preterm life, for which we studied impact of gestational age, birth weight, or birth weight Z-score on GSH synthesis rates on the second day of life.

Stable Isotope Infusion Protocol

[1-^{13}C]Glycine (99% enriched, sterility and pyrogenicity tested) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). On postnatal day 2 and 6 at 10:00 a.m., neonates received a primed (40 µmol/kg) continuous (20 µmol/(kg·h)) infusion of [1-^{13}C]glycine for 6 hours. Blood samples (400 µL each) were drawn from an indwelling arterial catheter after 4, 5, and 6 hours and collected in EDTA containing microtainers. Subsequently, samples were treated as described previously (14). Plasma and cell fractions were stored at -80°C until further analysis.

Glutathione Synthesis Rates and Precursor Amino Acid Concentrations

We measured GSH as total GSH, i.e. GSH + oxidized glutathione (GSSG), with a new technique using liquid chromatography isotope ratio mass spectrometry, described previously (14). For the analysis of ^{13}C- isotopic enrichment of glycine, we used gas chromatography – combustion – isotope ratio mass spectrometry, similar to an earlier developed method for measurement of the isotopic enrichment of threonine (15). The fractional synthesis rate of GSH (FSR_{GSH}) was measured according to the product/precursor- equation:

$$FSR_{GSH}\ (\%/d) = \frac{\text{slope}\, E_{[1\text{–}^{13}C]GSH_{t\,4,5,6}}}{E_{\text{intraerythrocytic}[1\text{-}^{13}C]\text{glycine}}} \times 24h \times 100\%$$

where E stands for enrichment expressed as mole percent excess (MPE). The nominator (product) represents the hourly increase of incorporated [1-^{13}C]glycine into GSH as calculated from the increase in enrichment between 4 and 6 hours of infusion. The denominator (precursor) represents the intraerythrocytic free [1-^{13}C]glycine enrichment at isotopic steady state. A steady state plateau was defined as an insignificant change with time in intraerythrocytic enrichment. Subsequently, the intravascular absolute synthesis rate of GSH (ASR_{GSH}) was calculated by the following equation:

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

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

Plasma concentrations of direct precursors of GSH (glutamate, glycine, and cysteine) as well as indirect precursors (glutamine, serine and methionine) were determined with a Biochrom 30 amino acid analyzer using ninhydrin detection. A reference range of term breast-fed infants is included (16).

Plasma Markers of Oxidative Stress

We measured advanced oxidized protein products (AOPP) in plasma according to the spectrophotometric assay described by Witko-Sarsat et al. (17). Tyrosine is oxidized to dityrosine in response to oxidative stress and can be considered a good endogenous marker. Experiments that expose protein to oxygen-free radicals have demonstrated the formation of dityrosine. Dityrosine has been recognized as an oxidative stress product of pathological response to disease or other environmental stress (18, 19).

Its concentration was measured according to the method described by Abdelrahum et al., based on liquid-liquid extraction, reversed-phase chromatography and fluorescence detection (20).

Statistical Analysis

Statistical analyses were performed using SPSS version 14.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). Data are expressed as means ± SD or as medians (min – max). Differences between study days were determined using paired samples T-tests. A P value of <0.05 was considered as statistically significant. Primary outcome of the study was the ASR_{GSH}. Based on an abstract (Shew S et al., Effects of cysteine on endogenous synthesis of taurine and glutathione by premature neonates receiving parenteral nutrition, 2000 PAS Annual Meeting, May 12-16, 2000, Boston, MA, USA, Abstract 1750), which showed an increase of 80% in ASR_{GSH} upon AA administration, we calculated that with an α of 0.05, a power of 0.80 and a difference in ASR_{GSH} of 0.22 mmol/(L·d) with an SD of 0.06, at least 3 infants were needed to detect a difference between day 2 and 6. We included 10 infants to increase power.

Results

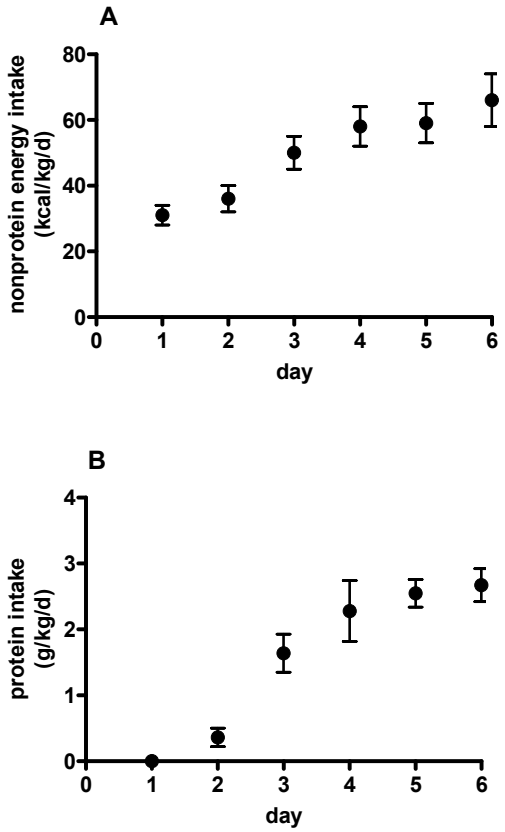
Subjects

In total, ten neonates were enrolled in the study. Birth characteristics and clinical details of the infants are shown in Table I. Non protein energy intake as well as protein intake (values derived from AA intake) are shown in Figure 1. Infants received vitamin C (10-12 mg/(kg·d)) and vitamin E (1.5 mg/(kg·d)) starting upon completion of study d2. The standard nutritional policy of our NICU dictated AA and lipid administration starting at 2:00 p.m. on the second day of life. As anticipated by study protocol, infants received AAs and lipids only after completion of study d2. All infants received ventilatory

Table I. Clinical characteristics. Values are expressed as means ± SD or median (min – max).

N (m:f)	10 (8:2)
Birth weight (g)	820 ± 134
Gestational age (wks)	26 4/7 ± 1 1/7
Birth weight Z-score (SD)	-1.2 ± 1.2
Apgar (5')	7 ± 3
CRIB	5 (1-8)

Figure 1. **(A) Actual parenteral and enteral nonprotein energy intakes (kcal/(kg·d)); (B) protein intakes (g/(kg·d)). Values are expressed as mean ± SD.**



support and additional oxygen during the six day period. The target SpO_2 range was set at 88 – 94%. According to our policy at the NICU, all infants being ventilated receive prophylactic antibiotics. Therefore, all infants included received antibiotics. Administration of these antibiotics were stopped after 48-72 hours whenever C-reactive protein concentrations were low and blood cultures were negative. During the study period, three infants suffered from intraventricular hemorrhage (grades II-III); eight infants received indomethacin treatment for patent ductus arteriosus.

Markers of Oxidative Stress

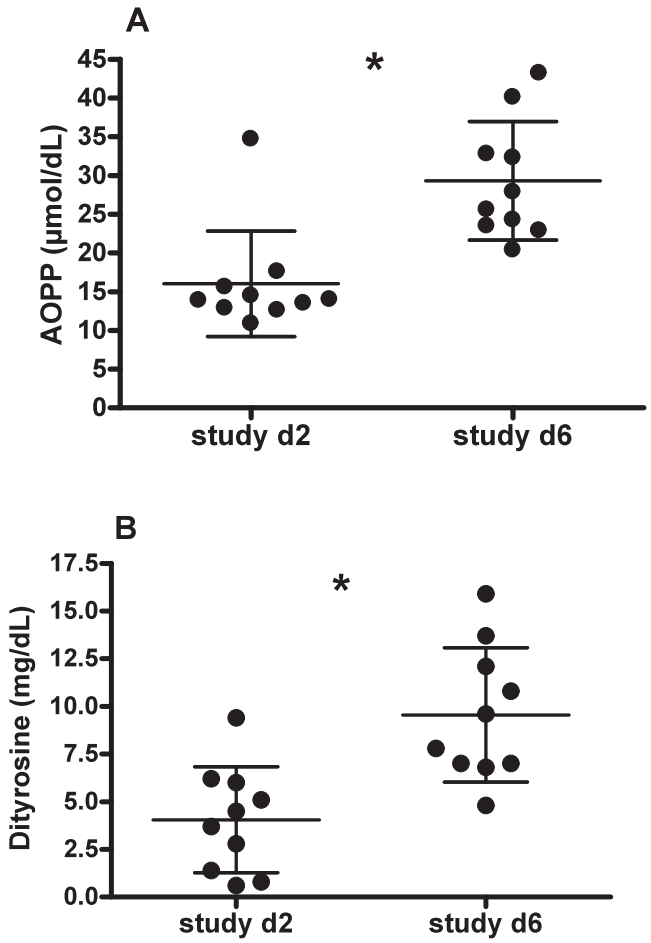
AOPP and dityrosine concentrations for individual subjects can be found in Figure 2. The mean concentrations of AOPP increased from day 2 towards day 6 (16.0 µmol/dL [SD: 6.8] vs 29.3 µmol/dL [SD: 7.6]; $P < 0.001$). A parallel increase was observed for dityrosine concentrations (4.0 mg/dL [SD: 2.8] vs 9.6 mg/dL [SD: 3.5]; $P = 0.003$).

We also calculated the ratio between tyrosine and dityrosine to correct for differences in total plasma protein concentrations (data not shown). However, in contrast to most other AAs, plasma tyrosine concentrations were lower on postnatal day 6, indicating an even higher relative increase in dityrosine load as compared to day 2.

Glutathione Kinetics on Day 2 and Day 6

Increased nutritional intake accompanied by increased postnatal age was followed by higher plasma concentrations of precursor AAs (Table II). We found, however, no difference in mean erythrocyte GSH concentration between day 2 and day 6 (1.67 mmol/L

Figure 2. (A) AOPP en (B) dityrosine concentrations at time of study d2 and study d6. Horizontal lines indicate mean and SD. *Statistically significant (p<0.05).



[SD: 0.40] vs 1.57 mmol/L [SD: 0.38], respectively; P = 0.62). Free intraerythrocytic [1-^{13}C]glycine enrichment reached a steady state after 4 hours of infusion. Mean enrichment at plateau was 3.9 MPE [SD: 1.0] during study d2 and 2.8 MPE [SD: 0.6] during study d6. In addition, we found a linear increase in GSH-bound [1-^{13}C]glycine enrichment between 4 and 6 hours of infusion.

Table II. Plasma concentrations (µmol/L) of AAs involved in GSH synthesis with a reference range of term breast-fed infants (36). Since cysteine is extremely susceptible to oxidation, it was measured as its cystine analogue. *p<0.05

Values are expressed as mean ± SD.

	study d2	study d6	term infants
glutamate	17 ± 6	53 ± 13*	24 – 243
glycine	187 ± 82	318 ± 135*	77 – 376
cystine	12 ± 7	23 ± 11*	35 – 69
glutamine	308 ± 193	479 ± 132	142 – 851
serine	91 ± 43	171 ± 45*	0 – 326
methionine	11 ± 7	22 ± 6*	22 – 50

In parallel with GSH concentrations, FSR_{GSH} did not increase from day 2 to day 6 (43 %/d [SD: 4] and 49 %/d [SD: 11], respectively; P = 0.16). Consequently, also ASR_{GSH} was not different (6.1 mg/(kg·d) [SD: 0.9] and 6.0 mg/(kg·d) [SD: 1.4], respectively; P = 0.76). Individual results are shown in Figure 3.

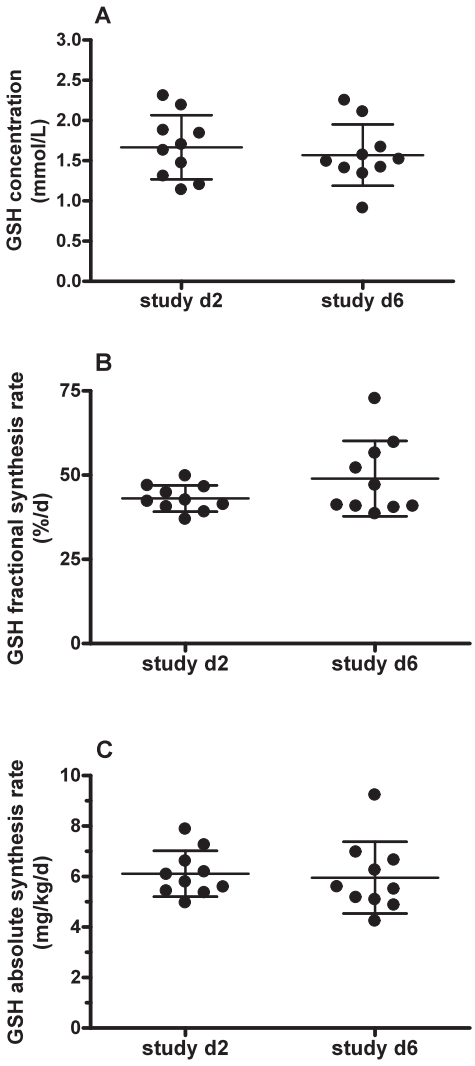
Birth weight was negatively correlated with FSR_{GSH} on postnatal day 2 (p=0.04), whereas gestational age was not. When birth weight was corrected for gestational age, i.e. birth weight Z-score, the correlation was more significant (p=0.006) (Figure 4).

Discussion

In this study, we showed that oxidative stress in ELBW infants is ongoing on day 6 of life, as reflected by higher AOPP and dityrosine concentrations compared to day 2. Despite increasing nutritional intakes towards day 6, resulting in higher plasma levels of precursor AAs, the erythrocytes of these infants did not appear to be exhibiting a response to a plasma oxidant load. Indeed, erythrocyte GSH concentrations and, more interestingly, synthesis rates did not increase.

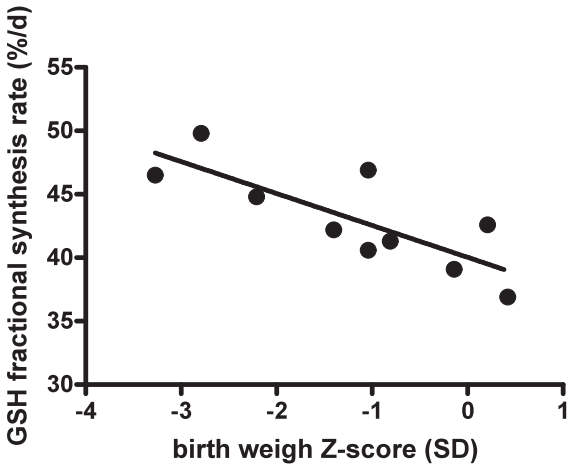
A possible explanation is that specific nutritional requirements, such as cyst(e)ine intake, might not be met. Ahola et al. reported earlier on the lack of increase in GSH concentration in the first week of life (13). The AA solution used in their study contained

Figure 3. (A) Glutathione concentrations; (B) fractional synthesis rates; (C) absolute synthesis rates. Horizontal lines represent mean and SD. There are no differences between both study days.



a modest amount of cysteine, which is assumed to be the rate limiting substrate for GSH synthesis, especially in preterm infants. Although cysteine administration in our study was higher, plasma concentrations remained low and possibly accounted for the lack of increase in erythrocyte GSH concentrations or synthesis rates. Within this context, it must be noted that AAs and glucose were administered separately, as glucose is

Figure 4. Correlation between glutathione fractional synthesis rate and birth weight Z-score (R2=0.63; p=0.006).



known to react with cysteine, forming its thiazolidine derivative which has decreased bioavailability (21).

Another cause for the lack of increase in erythrocyte GSH synthesis might be that requirements are highest in the direct postnatal phase, when the infant has to adapt to extrauterine life, and decrease subsequently. This theory is supported by a study in which we observed a significant rise in erythrocyte ASR_{GSH} on postnatal day 2 in infants who had received AAs directly from birth onwards (22).

Still, markers of protein oxidation were higher on postnatal day 6 as compared to day 2, as was observed earlier (5). It must be noted, however, that these protein oxidation markers were determined in plasma, whereas GSH kinetics were determined in erythrocytes. Plasma and erythrocytes may represent two different compartments reacting separately. On the other hand, rising AOPP and dityrosine concentrations might reflect cumulative oxidative damage resulting from the transition to the relatively hyperoxic extrauterine life and the subsequent ventilation with additional oxygen. It might also be due to prolonged administration of lipid infusion. Hong et al. recently demonstrated increased oxidative stress as reflected by augmented malondialdehyde concentrations in rabbit liver following 10 days of parenteral nutrition (23).

AOPP en dityrosine are not independent markers of oxidative stress as they both represent protein oxidation. Unfortunately, our mode of sample collection as well as the limitations posed to sampling volumes in preterm infants, did not allow us to determine alternative markers, such as isoprostanes or non protein bound iron.

We did not find a correlation between erythrocyte GSH synthesis rates and gestational age. However, we did find that infants with the lowest birth weight Z-score had the

highest FSR_{GSH}. Whether this is based on coincidence in this relatively small group, or is a stress related phenomenon accompanying low birth weight, these data confirm that *capacity* for GSH synthesis is not hampered in preterm infants. This is supported by data showing that preterm infants with gestational ages as low as 26 weeks demonstrate presence of GSH synthesizing enzymes independent of postnatal age (24, 25). On the other hand, in a study on GSH kinetics in healthy adults using the same method, we found an FSR_{GSH} of 62% in erythrocytes (22), which is considerably higher than values reported here for preterm infants, but similar to values for adults found by Jackson et al. and Lyons et al., although the latter determined GSH kinetics in whole blood rather than erythrocytes (26, 27). GSH concentrations, however, were lower in adults resulting in an ASR_{GSH} being only slightly higher than in these preterm infants. It must be noted, however, that adults were studied in the fasted state, which lasted the whole study period.

Besides de novo synthesis, availability of GSH is affected by recycling. Indeed, the lower FSR_{GSH} in erythrocytes of preterm infants might be explained by their increased recycling capacity of reconverting GSSG into GSH as compared to adults (28, 29). Recycling decreases the need for de novo synthesis and could explain, therefore, the lower FSR_{GSH} in preterm infants.

Gender was no criterion for inclusion. On the other hand, we must be aware that the resulting imbalance between boys and girls in our study might be a confounding factor, since sex differences have been described in GSH and cysteine metabolism (30, 31).

In this study we measured total erythrocyte GSH availability instead of individual concentrations of GSH and GSSG. A decreasing GSH/GSSG ratio is considered to be indicative of oxidative stress. GSH is, however, very susceptible to oxidation and not unlikely to cause artificial formation of GSSG (32). In our study, because of repeated blood sampling required for studying GSH kinetics, no blood was available to determine GSH/GSSG ratio, the more so because this would require procedures which interfere with our sample treatment necessary for GSH synthesis measurements.

We studied GSH metabolism in erythrocytes, which are readily available as opposed to other tissues. Though antioxidant concentrations or kinetics at organ specific sites might be considered as more clinically relevant at first sight, it has been recognized over the years that erythrocytes not only reflect, but also significantly contribute to overall antioxidant defense (33-35).

We conclude that oxidative stress is ongoing in preterm infants in the first week of life, as reflected by markers of oxidative stress. Despite increased substrate availability on day 6 as compared to day 2, GSH synthesis rates did not increase. Future research should be directed towards defining whether additional specific substrates, such as cysteine, might stimulate GSH production.

Acknowledgements

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8 Glutathione synthesis rates following amino acid administration directly following birth in preterm infants

Frans WJ te Braake, Henk Schierbeek, Karien de Groof, Andras Vermes, Mariangela Longini, Giuseppe Buonocore, and Johannes B van Goudoever

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Abstract

Availability of glutathione, the main intracellular antioxidant, is compromised in preterm neonates. A possible explanation is low substrate availability for synthesis, as many neonatologists for fear of intolerance are reluctant to administer amino acids in the direct postnatal period.

The objective of the study was to determine effects of amino acid administration directly following birth on glutathione synthesis rates and markers of oxidative stress.

Premature infants (<1500 g) received from birth onwards either dextrose (control group, n=10) or dextrose and amino acids (2.4 g/(kg·d)) (intervention group, n=10). On postnatal day 2, [1-^{13}C]glycine was administered to determine glutathione fractional synthesis rates (FSR_{GSH}) and absolute synthesis rates (ASR_{GSH}) in erythrocytes. In plasma, advanced oxidized protein products (AOPP) and dityrosine, both markers of oxidative stress, were determined. Results are expressed as mean ± SD.

The FSR_{GSH} was not different between groups (44 ± 6 and 48 ± 9 %/d in the control and intervention group respectively (p=0.28)). The concentration of erythrocyte glutathione in the intervention group (2.28 ± 0.35 mmol/L) was higher than in the control group (1.73 ± 0.37) (p<0.001). The ASR_{GSH} was 6.5 ± 1.5 and 11.3 ± 1.9 mg/(kg·d) in the control and intervention group, respectively (p<0.001).

AOPP and dityrosine concentrations were not different between groups.

In conclusion, amino acid administration directly following birth increases ASR_{GSH} in preterm infants. Our data are consistent, however, with higher glutathione concentration rather than a higher FSR_{GSH}. Greater availability of glutathione, nevertheless, did not bring down markers of oxidative stress.

Introduction

Birth coincides with a sharp increase in oxygen exposure. The formation of reactive species, such as superoxide, hydrogen peroxide and hydroxyl radicals evokes up-regulation of antioxidant defense systems in full term infants (1) or rabbits (2). This, however, does not seem to occur in preterm infants, as reflected by poor antioxidant availability and presence of protein and lipid (per)oxidation products (3, 4). Yet, upon their unanticipated transition to the extrauterine world, preterm neonates frequently receive, though not necessarily require, ventilation with high concentrations of oxygen. This may result in oxidative stress, which is strongly associated with neonatal diseases such as bronchopulmonary dysplasia, retinopathy of prematurity, and periventricular leukomalacia (5-8).

With concentrations in the millimolar range, glutathione (GSH) is the most important intracellular antioxidant. Most cells are equipped with the enzymatic machinery to synthesize this tripeptide of glutamate, cysteine and glycine. Moreover, the enzymatic apparatus is present and active already in the second trimester of pregnancy and thus not a limiting factor for GSH synthesis in preterm infants (9, 10). GSH concentrations in erythrocytes and plasma of preterm infants are high immediately after birth, but then drop to significantly lower levels than found in term neonates in the neonatal period (3, 11). This shortage may be due to the fact that preterm infants do not tolerate significant amounts of enteral nutrition in the first days of life, and are, therefore, given parenteral nutrition, typically starting off with dextrose only. Meanwhile, however, the safety of early amino acid (AA) administration in preterm infants has been well established, as relevant studies found no abnormal blood gas values or abnormal plasma AA profiles (12, 13). In addition, infants' catabolic state when receiving dextrose only was found to convert into an anabolic state representing true growth upon AA administration (14). Despite these findings, AA administration directly after birth is still not uniformly standard of care.

We hypothesized that GSH production in preterm infants is compromised by shortage of substrates, and that AA administration will stimulate GSH synthesis rates. To test this hypothesis, we conducted a stable isotope study designed to determine synthesis rates of GSH in infants receiving either dextrose only or dextrose and AAs. Degree of oxidative stress was established by measuring concentrations of advanced oxidized protein products (AOPP) (15) and dityrosine (16, 17), both markers of oxidative stress. We hypothesized that preterm infants receiving dextrose only would show the highest concentrations of AOPP and dityrosine.

Methods

Design

The study was designed as a randomized clinical trial performed in 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 protocol was approved by the Erasmus MC Medical Ethical Committee and informed written parental consent was obtained prior to the study.

Subjects

Subjects were premature infants with a birth weight <1500 g, born in the Erasmus MC–Sophia Children's Hospital, had an indwelling arterial catheter for clinical purposes, and were expected to be completely dependent on parenteral nutrition for the first 2 days of life. Directly after birth they were randomly assigned to receive either only dextrose during the first 2 days (control group), or dextrose and AAs (2.4 g/(kg·d) (Primene 10%, Baxter, Clintec Benelux N.V., Brussels, Belgium) within 2 hours postnatally (intervention group). The composition of the AA solution can be found in Table I. AA and dextrose solutions were infused constantly without interruptions during the study. Lipids were not administered until after the study period. Exclusion criteria included erythrocyte transfusions within 12 hours prior to the study or during the study, known congenital abnormalities, chromosome defects, and metabolic, endocrine, renal, or hepatic disorders. For all infants, we recorded birth weight, gestational age, birth weight Z-scores, severity of illness at entry of the study by means of Apgar and CRIB scores (18). We recorded plasma AA concentrations, and caloric intake and AA intake. In addition, we recorded fractions of inspired oxygen, blood glucose levels, and incidence of sepsis as evidenced by bacteremia. According to our policy at the NICU, all infants being ventilated receive prophylactic antibiotics. Therefore, all infants included received antibiotics. Administration of these antibiotics are stopped after 48-72 hours whenever C-reactive protein concentrations are low and blood cultures are negative.

Tracer infusion protocol and sample collection

[1-^{13}C]Glycine (99% enriched, sterility and pyrogenicity tested) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and was dissolved in a 0.9% saline solution, filtered (0.2 μm) and sterilized by the hospital's pharmacy. The final product was tested for identity, content, sterility and pyrogenicity.

On postnatal day 2 neonates received a primed (40 μmol/kg) continuous (20 μmol/(kg·h)) infusion of [1-^{13}C]glycine for 6 hours. Blood samples (400 μL each) were drawn

Table I. Composition of the amino acid solution Primene 10% (Baxter, Clintec Benelux N.V., Brussels, Belgium)

Amino acid	g/L
L-Isoleucine	6.70
L-Leucine	10.00
L-Valine	7.60
L-Lysine	11.00
L-Methionine	2.40
L-Phenylalanine	4.20
L-Threonine	3.70
L-Tryptophane	2.00
L-Arginine	8.40
L-Histidine	3.80
L-Alanine	8.00
L-Aspartate	6.00
L-Cysteine	1.89
L-Glutamate	10.00
Glycine	4.00
L-Proline	3.00
L-Serine	4.00
L-Tyrosine	0.45
L-Ornithine-HCl	3.18
Taurine	0.60

from an indwelling arterial catheter after 4, 5, and 6 hours and collected in EDTA containing microtainers to quantify erythrocyte free glycine enrichment, GSH-bound glycine enrichment, GSH concentration, plasma oxidative stress markers, and plasma GSH precursor AA concentrations.

Samples were immediately put on melting ice after centrifugation at 3500 x G for 10 min at 4°C. After the plasma fraction was removed, the lower layer containing primarily erythrocytes was reconstituted to its original volume with ice-cold distilled water to disrupt cell membranes. The plasma and cell fractions 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 measure fractional and absolute synthesis rates. For this purpose, we used a new technique described previously, using an LC-Isolink® interface (Thermo Electron, Bremen, Germany) coupled to a Delta XP isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany) (LC-IRMS) (19). This highly sensitive method requires only a very small sample volume, whereas no derivatization is required. Briefly, erythrocytes were disrupted by freezing and thawing. Next, 40 µL of a mixture of 0.2 mg/mL norleucine and 1.0 mg/mL norvaline was added as internal standards and the samples were deproteinized by adding 200 µL of 6 % g/v perchloric acid, left on ice for 10 minutes and finally centrifuged at 10.000 x G for 20 minutes. The supernatant was transferred to a clean tube and the pH was adjusted to 8 - 9 with a KOH (4 mol/L) solution. Excess perchloric acid was precipitated and removed by centrifugation at 10.000 x G for 10 minutes. 100 µL of $NaHPO_4$ (0.5 mol/L) was added to maintain pH 9. The supernatant was filtered through a 0.2 µm Nylon membrane filter (Nylon, Alltech, Breda, The Netherlands). An aliquot of 150 µL was transferred to a sample vial and 20 µL was injected for each analysis of both GSH concentration and ^{13}C-isotopic enrichment by LC- IRMS. The remaining supernatant was used for analysis of ^{13}C- isotopic enrichment of glycine by gas chromatography – combustion – isotope ratio mass spectrometry, similar to an earlier developed method for measurement of the isotopic enrichment of threonine (20). 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).

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. It was measured according to the product/precursor- equation:

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

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

cant change with time in intraerythrocytic enrichment. Subsequently, the intravascular absolute synthesis rate (ASR_{GSH}) was calculated by the following equation:

ASR_{GSH} (mg/(kg·d)) = FSR_{GSH}/100 x conc x 307 x ht x 0.075

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

Oxidative stress markers

We measured AOPP in plasma by the spectrophotometric assay described by Witko-Sarsat et al (15). Dityrosine concentrations were measured by the method described by Abdelrahim et al., based on liquid-liquid extraction, reversed-phase chromatography and fluorescence detection (16).

Glutathione kinetics in healthy adults

Since kinetic measurements require venous access and an indwelling arterial catheter, healthy term neonates cannot serve as controls. GSH kinetics so far have only been studied in older infants and adults, as relatively high blood volumes are needed for precise determination of enrichment and concentration. In two studies on GSH kinetics in healthy adults, mean FSR_{GSH} was found to be 65%/d and 83%/d, respectively (21, 22). GSH concentrations, however, were found to vary substantially between studies (23). Even minimal manipulation of samples can result in loss of GSH, thereby creating false assumptions with respect to the in vivo situation. To correct for different methods yielding different results, we determined GSH kinetics in healthy adults by exactly the same method we used for preterm infants. Studies were conducted after an overnight fast, and subjects remained in the fasting state throughout the study.

Statistics

Statistical analyses were performed using SPSS version 15.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). Data are expressed as means ± SD or as medians (min – max). Primary outcome of the study was the glutathione fractional synthesis rate. Based on an abstract by Shew et al. (24), we calculated that with an α of 0.05, a power of 0.80 and a difference in ASR_{GSH} of 0.22 mmol/(L·d) with an SD of 0.06, group size needed to be at least 3 to detect a difference. We included 10 infants in each group in order to increase power.

Differences between groups were determined using independent t-tests or Mann-Whitney tests in case of normal or skewed distribution of the study groups, respectively. A P value of <0.05 was considered as statistically significant.

Results

Clinical characteristics are listed in Table II.

All infants received additional oxygen as part of their treatment. We found no correlation between fractions of inspired oxygen and GSH concentration or FSR_{GSH}.

Table II. Clinical characteristics[1]

	control	intervention
N (M:F)	10 (7:3)	10 (4:6)
birth weight (g)	990 ± 205	916 ± 150
gestational age (wks)	27 1/7 ± 2	275/7 ± 21/7
birth weight Z-score (SD)	-0.4 ± 1.3	-1.4 ± 0.9
mode of delivery vaginal:cesarean section	5:5	5:5
Apgar score (5 min)	8 (6 – 10)	7 (4 – 9)
CRIB score[2]	5 (1 – 7)	4 (1 – 8)
FiO2 minimum	21 (21 – 29)	21 (21 – 24)
maximum	54 (24 – 100)	45 (28 – 72)

[1] Values are expressed as either mean ± SD or median (min-max).

There were no statistical differences between groups (Student's T-test or Mann-Whitney, depending on distribution).

[2]The CRIB score (Clinical Risk Index for Babies) indicates the degree of illness. The score is positively correlated with the severity of illness.

Nutritional intakes before and during the study are shown in Table III.

Table III. Nutritional intakes before and during the study[1]

	control (n=10)	intervention (n=10)	P value
nonprotein energy intake (kcal/(kg·d))	38 ± 4	42 ± 8	0.22
amino acid intake (g/(kg·d))[2]	0 ± 0	2.5 ± 0.1	<0.001

[1] Values are expressed as mean ± SD (Student's T-test).

[2] As anticipated by study design, the control group did not receive amino acids during or prior to study.

Blood glucose levels were not different between groups and none of the infants received insulin during or prior to the study (data not shown). Also, there were no differences in incidence of hyperglycemia.

In one blood culture micrococcus species was isolated, which was considered contamination. None of the other infants had either a rise in C-reactive protein concentrations or positive blood cultures before or during the study.

Concentrations of plasma precursor AAs

Table IV shows plasma concentrations of all AAs involved in GSH synthesis with reference values obtained from healthy term breast-fed infants (25); AA concentrations were

Table IV. Plasma concentrations of amino acids[1,2]

	control (n=10)	intervention (n=10)	P value	term infants
glutamate	19 ± 5	72 ± 47	0.017	76 – 551
cystine	14 ± 9	27 ± 10	0.011	33 – 55
glycine	206 ± 71	341 ± 154	0.047	66 – 432
glutamine	280 ± 167	566 ± 243	0.012	147 – 623
serine	94 ± 31	189 ± 91	0.022	79 – 227
methionine	12 ± 5	36 ± 24	0.026	21 – 55

[1] Amino acids that are directly or indirectly involved in glutathione synthesis with a reference range of term breast-fed infants(25).

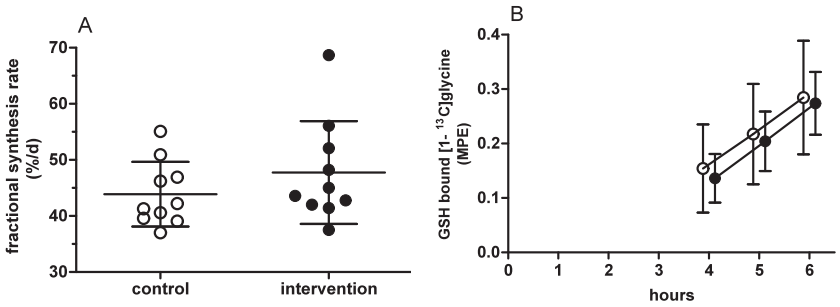
[2] Values are expressed as mean ± SD (Student's T-test).

significantly higher in the intervention group. Concentrations of glutamate, cystine, and methionine in the control group were below reference ranges. Although the AA solution administered contained cysteine (1.89 g/L), cystine concentrations still were low in the intervention group as well.

GSH concentrations and synthesis rates

In Figure 1 precursor and product enrichments are plotted against duration of tracer infusion. Free intraerythrocytic [1-^{13}C]glycine, considered as the precursor for GSH synthesis, reached a plateau after 4 hours of infusion defined as no significant increase in enrichment. Precursor enrichments did not differ between the groups (3.6 ± 0.7 MPE in each). Levels of [1-^{13}C]glycine bound to GSH linearly increased between 4 and 6 hours of infusion, with a mean R^2 of 0.996 and 0.994 between the values measured for the control and intervention group, respectively. The LC-IRMS method used in this study

Figure 1. Means and SD's of isotopic steady state of [1-13C]glycine (precursor) enrichments (A) and [1-13C]glutathione (product) enrichments (B) in the control (○, n=10) and intervention (●, n=10) groups. There were no significant differences in either isotopic steady state of [1-13C]glycine enrichment or increase in [1-13C]glutathione enrichment (Student's T-test).



quantified [1-^{13}C] enrichment of the total GSH pool, since IRMS is known to combust all carbon elements of GSH into CO_2. We, therefore, were not able to discriminate between [1-^{13}C] enrichment of GSH originating from glycine or cysteine. We studied, however, the cysteine peak in our chromatogram, and we did not find any enrichment, thereby excluding possible overestimation of the FSR.

GSH kinetic data are shown in Figure 2. FSR_{GSH} did not differ between groups. The concentration of erythrocyte GSH in the intervention group was higher, however, than that in the control group. As a result, also ASR_{GSH} was higher in the intervention group.

Plasma levels of oxidative stress markers

Results are shown in Figure 3. We found no differences between groups for both markers. These results are in agreement with earlier studies on AOPP levels in preterm infants and indicate the presence of oxidative stress (4, 26). Increased GSH availability as a result of AA administration did not result in lower AOPP levels on postnatal day 2.

GSH kinetics in healthy adults

We included 5 healthy non smoking adults. They had an age of 34 ± 8 years and a Body Mass Index of 22.5 ± 1.2 kg/m^2 (mean ± SD).

We found a mean concentration of 1.43 ± 0.13 mmol/L which is comparable to the concentrations reported by Darmaun and colleagues (22). The mean FSR_{GSH} in erythrocytes was 62 ± 2 %/d resulting in a mean ASR_{GSH} of 7.7 ± 1.1 mg/(kg·d). Thus, the ASR_{GSH} of fasted adults is lower than that of fed preterm infants.

Figure 2. **Glutathione fractional synthesis rates (A), concentrations (B) and absolute synthesis rates (C) in erythrocytes in the control (○, n=10) and intervention (●, n=10) groups expressed as individual cases with horizontal lines representing means and SD's. There were no significant differences in fractional synthesis rates between groups (Student's T-test). Glutathione concentrations and absolute synthesis rates were higher in the dextrose + AA group (P<0.001 for both concentration and absolute synthesis rate, Student's T-test).**

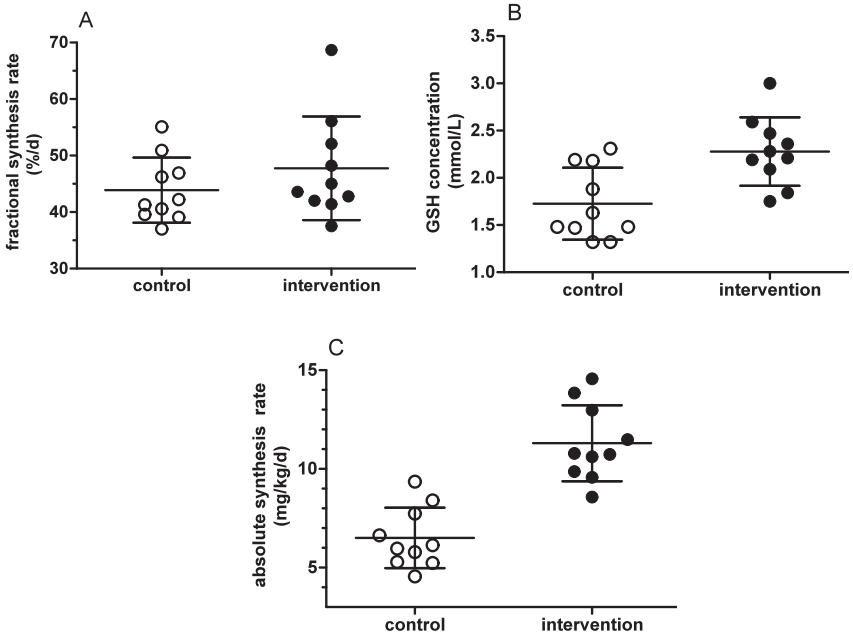
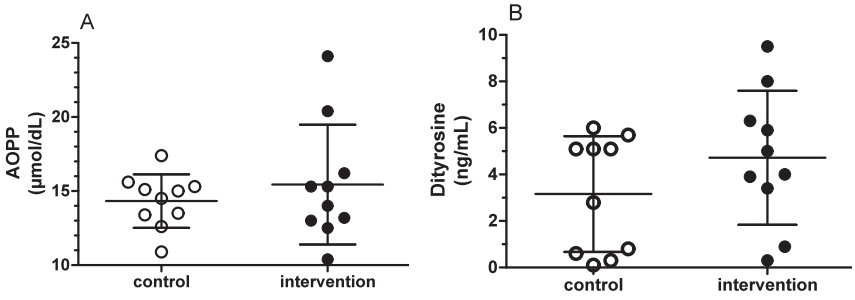


Figure 3. **Plasma concentrations of advanced oxidized protein products (AOPP) (A) and dityrosine (B) in the control (○, n=10) and intervention (●, n=10) groups expressed as individual cases with horizontal lines representing means and SD's. There were no statistically significant differences for either AOPP or dityrosine concentrations (Student's T-test).**



Discussion

We showed AA administration to be a safe, simple and efficacious means of increasing GSH synthesis rates. We demonstrated a 32% increase in erythrocyte GSH concentration and a 74% increase in the ASR_{GSH} following administration of 2.4 g AAs/(kg·d) as compared to dextrose administration solely. These results clearly bring out the discrepancy between, on the one hand, demands of the body and, on the other hand, the scarce availability of substrates if only dextrose is administered. Antioxidant defense build-up was not associated, however, with lower concentrations of oxidative stress markers.

The observed increase of the ASR_{GSH} can almost exclusively be attributed to the increase in concentration. This is fascinating, since in adults and children alike increases in ASR_{GSH} seem to arise primarily from increases in FSR_{GSH} rather than from elevated concentrations (21, 27, 28). We have two explanations. For one, the FSR_{GSH} might have been increased already on the first day of life, and then have dropped as a result of negative feedback. A study performed on the first day of life under the same conditions could provide more insight. Second, the lack of increase in FSR_{GSH} might be related to the function of GSH as an AA reservoir. Plasma glycine concentration rose 65% after AA administration (Table III). It follows that plasma glycine enrichment must have been lower in the intervention group, since infusion rates of [1-^{13}C]glycine were identical for both groups. Despite theoretically lower plasma glycine enrichment in the intervention group as compared to the control group, intraerythrocytic enrichment did not differ between groups, as shown in Figure 2. Seeing that GSH itself is the major intraerythrocytic pool of glycine, the most plausible explanation is decreased breakdown of intracellular GSH in the intervention group compared to the control group, as proposed earlier (29). Note that GSH is an important AA reservoir, its intracellular concentration being in the millimolar range whereas the free fractions of its constituent AAs, especially cysteine, are in the micromolar range. Altogether, these data strongly suggest decreased consumption of GSH in the intervention group, possibly resulting – as we showed recently – from increased synthesis of other antioxidants such as albumin (30).

An explanation for the overall low FSR_{GSH} in preterm neonates as compared to adults is increased recycling of GSSG to GSH in neonates. Normally, GSSG concentrations are kept very low to protect the cell from a shift in redox equilibrium. This is achieved by either reducing GSSG to GSH or exporting it to the extracellular space, dependent on the availability of the enzyme glutathione reductase and NADPH (31). Indeed, increased recycling of GSSG into GSH in erythrocytes of preterm neonates as opposed to adults was observed earlier (32, 33). More efficient recycling could very well decrease the need for de novo synthesis and perfectly fits our data, i.e. a relatively low FSR_{GSH} in preterm infants as compared to adults.

Blood glucose concentrations and incidence of sepsis were not different between groups. This is relevant, seeing that both hyperglycemia and sepsis are known to produce oxidative stress (34, 35).

The importance of GSH in maintaining health and preventing oxidative stress has been widely studied. Chessex et al. studied the individual effects of hyperoxia and nutrient restriction on liver and lung GSH availability in preterm guinea pigs (36). They concluded that total parenteral nutrition not only increased both liver and lung GSH concentrations but also protected against hyperoxic lung injury and associated mortality. In agreement, Welty et al. and Yeung et al. recommended administration of antioxidants or their precursors as soon as possible following birth in order to prevent oxidative stress related diseases, such as bronchopulmonary dysplasia (37, 38).

GSH kinetics were measured using a new technique which transforms all GSH to its dimeric form (GSSG). We did not, therefore, discriminate between GSH and GSSG. However, since the fractional synthesis rate is a relative measure, it will not be influenced by a difference in redox state. We measured GSH kinetics in erythrocytes, which are readily accessible as opposed to other tissues. Moreover, erythrocytes can protect other tissues, such as the lung, by providing intracellular antioxidants (39) or by directly taking up ROS (40). In a very recent study, Giustarini et al. provided strong evidence for a role for erythrocytes as GSH donor for other tissues (41). They demonstrated active GSH export towards the plasma, indicating that, besides the liver, erythrocytes might significantly contribute to the extracellular GSH pool. These studies, however, fail to elucidate the mechanism by which the GSH is exported, since GSH is assumed unable to cross the cellular membrane intact.

We measured AOPP and dityrosine as markers of oxidative stress. Tyrosine is oxidized to dityrosine in response to oxidative stress and can be considered a good endogenous marker. Experiments that expose protein to oxygen-free radicals have demonstrated the formation of dityrosine. Dityrosine has been recognized as an oxidative stress product of pathological response to disease or other environmental stress (17, 42).

The AOPP concentrations were higher than those found in older, more stable preterm infants as well as healthy adults (4, 15).

Cysteine is generally assumed to be the rate limiting substrate for GSH synthesis. It is also considered to be an essential AA in the preterm neonate on the grounds of high cystathionine concentrations (43) and low cystathionase activity impeding the conversion of methionine into cysteine (44). Although evidence is mounting that preterm infants are in fact able to synthesize cysteine (45, 46), demands may still exceed capacity to synthesize. Indeed, earlier we showed that plasma cyst(e)ine concentrations were below reference values, both in group not receiving any AAs and in a group receiving AAs including cysteine (14). The AA solution we used provides methionine and cysteine,

delivered as cysteine-HCl. Premixed AA solutions can only contain modest amounts of cysteine-HCl due to instability of cysteine at higher pH.

We demonstrated that AA administration to preterm infants directly postnatally is a safe and efficient way to increase GSH synthesis rates. Our data suggest that this does not increase GSH consumption, as intracellular GSH breakdown seems to decline. Levels of oxidative stress markers nevertheless remained high. Plasma cystine concentrations rose upon AA administration, but still remained low. Worthy of further research is the question whether higher doses of AAs, or additional cysteine in particular, would further increase GSH synthesis rates.

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9 Glutathione synthesis rates in the immediate postnatal phase

Denise Rook, Frans WJ te Braake, Henk Schierbeek, Mariangela Longini, Giuseppe Buonocore, and Johannes B van Goudoever

Submitted

Abstract

Preterm infants have diminished antioxidant defense mechanisms. Although glutathione (GSH) concentration, the main intracellular antioxidant, increases upon amino acid (AA) administration in preterm infants, the fractional synthesis rate of GSH (FSR_{GSH}) was not increased on day 2. We hypothesized that GSH synthesis is already upregulated directly postnatally, with a subsequent negative feedback on day 2.

The objective was to determine GSH synthesis rates on day 1 or day 2 in preterm infants receiving AAs directly following birth.

GSH synthesis rates were measured using stable isotope techniques in very low birth weight (VLBW) infants on postnatal day 1 or 2. Advanced oxidized protein products (AOPP) were determined to quantify oxidative stress. Results are expressed as mean ± SD.

Eighteen infants (mean birth weight of 988 gram and gestational age of 27 $^{6}/_{7}$ weeks) were studied at either 7 or 31 hours postnatally. 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 between groups. In contrast to the hypothesis, the absolute synthesis rate of GSH (ASR_{GSH}) tended to be higher 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.

In conclusion, GSH concentration in VLBW infants on postnatal day 2 is significantly higher than that on postnatal day 1. A concomitant increased synthesis rate on day 1 was not found, suggesting that GSH consumption decreases upon AA administration.

Introduction

The sudden increase in oxygen pressure accompanying birth results in the increased formation of reactive oxygen species (ROS). Furthermore, 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 up 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 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 is increased during the last days of pregnancy (4). So when born premature, most antioxidant defense mechanisms function suboptimal at birth. The resulting redox imbalance causes oxidative stress, which is thought to be instrumental in the pathogenesis of the so-called 'Oxygen Radical Disease in Neonatology', which 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 concentration falls rapidly, however, immediately after birth in preterm infants. Recently, we demonstrated that amino acid (AA) administration to preterm infants from birth onwards results in a higher GSH concentration on day 2, when compared to the levels found in infants receiving dextrose only (13). This was, however, not accompanied by a higher fractional synthesis rate (FSR) on day 2. We, therefore, hypothesized that GSH synthesis is already upregulated directly after birth, and that negative feedback subsequently lowers GSH synthesis rate on postnatal day 2. The purpose of the present study was to reveal the mechanism behind the increased availability of GSH with early administration of AAs. To this aim we conducted a stable isotope technique to determine glutathione synthesis rates on postnatal day 1 or 2 in infants receiving AAs directly following birth.

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

Methods

This study was designed an observational clinical trial in which infants were randomized to be studied either on postnatal day 1 or 2. 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 dextrose 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 dextrose 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 or on day 2. [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 GSH fractional synthesis rates (FSR_{GSH}) in erythrocytes. Different infants were used for the tracer infusion study on either day 1 or day 2, because of the washout time of [1-^{13}C]glycine.

Sample analyses

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. In short, erythrocytes were disrupted by freezing and thawing. Then 40 µl of a mixture of 0.2 mg/ml norleucine and 1 mg/ml norvaline was added as internal standards and the samples were deproteinized by adding 200 µl of 6 % g/v perchloric acid, left for 10 min on ice and finally centrifuged at 10'000 G for 20 min. The supernatant was transferred to a new tube and the pH was adjusted to 8 - 9 with 4 Molar KOH. Excess of perchloric acid was precipitated and removed by centrifugation at 10'000 G for 10 min. 100 µl of 0.5M NaHPO4 was added to maintain the pH at 9. The supernatant was filtered through a 0.2 µm Nylon membrane filter (Nylon, Alltech, Breda, The Netherlands). An aliquot of 150 µl was transferred in a sample vial and 20 µl was injected for analysis of both GSH

concentration and 13C- isotopic enrichment by LC-C-IRMS. Analysis was performed in duplicate. The remaining supernatant was used for analysis of ^{13}C- isotopic enrichment of glycine, for which we adapted our earlier developed method on the derivatization of threonine and measurement with GC-C-IRMS (19, 20).

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.

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

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 absolute synthesis rate of GSH (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·d) = FSR_{GSH}/(100 x conc. x 307 x ht x 0.075)

where conc. is 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. In test wells, 40 µL of plasma was diluted with 120 µL PBS and transferred to a 96-well microtiter plate, followed by addition of 20 µL of acetic acid. In standard wells, 10 µL of 1.16 M potassium iodide were added to 200 µL of chloramine–T solution (0–100 µmol/L) followed by 20 µL of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm on the microplate reader against a blank containing 200 µL of PBS, 10 µL of potassium iodide, and 20 µL of acetic acid. AOPP measurements were performed in duplicate. 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 absolute synthesis rate. Based on earlier research from our research group (13), we calculated that with an α of 0.05, a power of 0.80 and a difference in ASRGSH of 4.5 mg/(kg·d) with an SD of 1.9, group size needed to be at least four to detect a difference. We included 8 and 10 infants in each group in order to increase power. 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 or day 2 (7 ± 4.8 and 31 ± 5.9 hours after birth, respectively). Clinical characteristics are displayed in Table I. 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 II.

Table I. Clinical characteristics

	Day 1	Day 2
N (male : female)	8 (7:1)	10 (8:2)
Gestational age (wks)	28 2/7 ± 4/7	27 4/7 ± 2 0/7
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 score2	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)
FiO2 minimum (%) on day 1	21 (21-25)	21 (21-21)
maximum (%) on day 1	30 (21-45)	55 (29-100) *
FiO2 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 FiO2 were significantly different between groups. Other characteristics were not different between groups

* $p < 0.05$

1The CRIB score (Clinical Risk Index for Babies) indicates the degree of illness. The score has a maximum of 23 point and is positively correlated with the severity of illness.

Table II. Nutritional intakes on each study day. As anticipated, infants received increased caloric intake on day 2 compared to day 1.

	Day 1	Day 2
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 III shows the plasma concentrations of all AAs involved in GSH synthesis with reference values obtained from healthy term breast-fed infants (21). Cystine concentration on day 2 was significantly lower than that on day 1 and was below the reference value (21). Concentrations of glutamate, glutamine, methionine and serine were not different between the groups, although glutamate concentrations were below reference values.

Table III. Plasma AA concentrations in µmol/L. AA concentrations for one patient of the day 2 group could not be determined because of shortage of plasma.

	Term healthy infants[1]	Preterm Day 1 (n=8)	Preterm Day 2 (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).

[1]Reference levels of plasma amino acid concentrations in healthy term breast-fed infants

* $p < 0.002$

GSH concentrations and synthesis rates

Free intraerythrocytic [1-^{13}C]glycine enrichments reached a plateau after 4 hours of infusion (Figure 1). Enrichments did not differ between the groups (mean 3.9 ± 0.9 and 3.4 ± 0.5 MPE in Day 1 and Day 2 group, respectively, $p = 0.18$).

GSH kinetic data are shown in Figure 2. The concentration of erythrocyte GSH on day 2 was significantly higher than that on day 1. However, FSR_{GSH} was not different between groups. Also, the ASR_{GSH} did not differ between the groups although there was a trend towards increased ASR_{GSH} on day 2 ($p=0.054$).

Figure 1. Means and SD's of isotopic steady state of intracellular [1-13C]glycine (precursor) enrichments expressed as mole percent excess on Day 1 (■, n=8) and Day 2 (□, n=10). There were no significant differences in isotopic steady state of [1-13C]glycine enrichment (Mann-Whitney test).

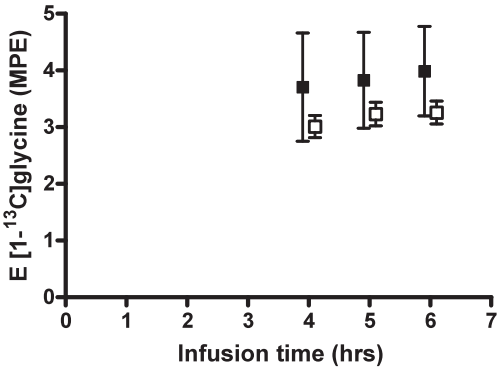
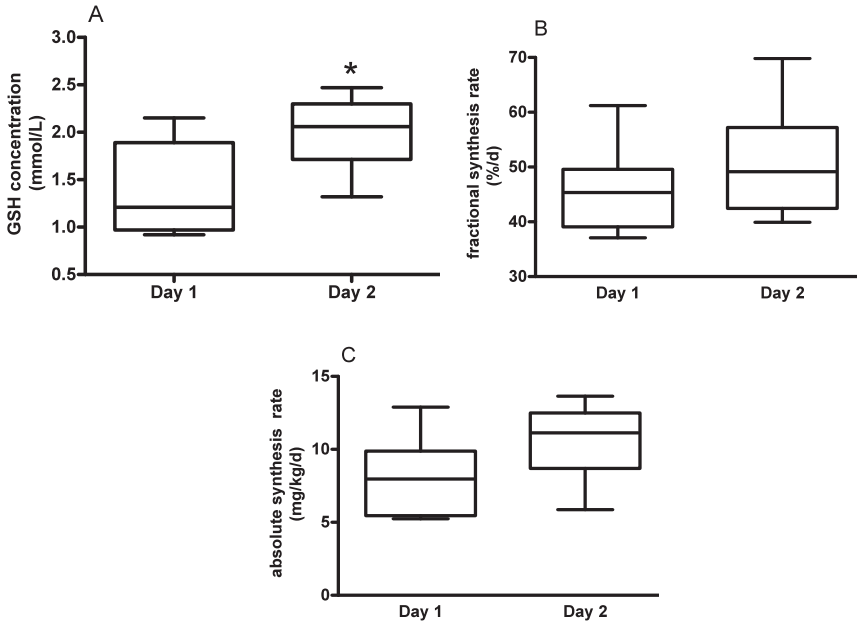


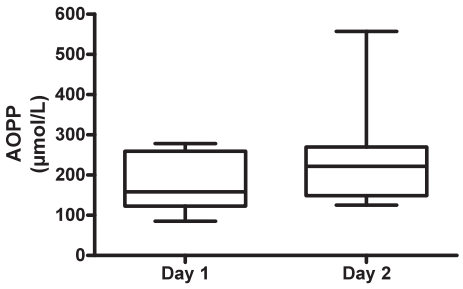
Figure 2. **Glutathione concentrations (A), fractional synthesis rates (B) and absolute synthesis rates (C) in erythrocytes on day 1 (n=8) and day 2 (n=10) expressed as box-plots. Glutathione concentrations on day 2 were significantly higher than those measured on day 1 (* p<0.05, Student's T-test). There were no significant differences in fractional synthesis rates and absolute synthesis rates between groups (Student's T-test).**



Plasma levels of AOPP

Results are shown in Figure 3. No differences were found between groups for plasma concentration of AOPP. The mean plasma concentration of AOPP of both groups was 217

Figure 3. **Plasma concentrations of advanced AOPP (expressed as µmol/L chloramine-T equivalents) in preterm infants on day 1 (n=8) and day 2 (n=10). Data are expressed as box-plots. There were no statistically significant differences in AOPP concentrations between groups (Student's T-test).**



± 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, 22, 23).

Discussion

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 an increased inspired oxygen fraction on day 2. One would expect that the higher inspired oxygen fraction would cause more reactive oxygen species, which subsequently would decrease GSH concentrations or upregulate GSH synthesis.

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, though without a concomitant rise in GSH fractional synthesis (13). We, therefore, speculated that GSH synthesis is already increased immediately after birth and that the increased GSH concentration subsequently down regulates synthesis on day 2 through negative feedback. In the present study, however, the fractional synthesis rate was not increased on the first day after birth, whereas the GSH concentration was still low. There was even a trend towards a higher absolute synthesis rate on day 2. It would seem, therefore, 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 (24). The method for measuring GSH concentration in the present study does not discriminate between reduced and oxidized GSH. Therefore, the increased total GSH concentration on day 2 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 (25, 26). Consequently, the increased GSH concentration is likely to be caused by decreased consumption of GSH after early AA administration in preterm infants.

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) (27). It is possible that the latter two AAs brought about the antioxidant effect. Previous studies indeed showed that methionine residues may protect proteins from critical oxidative damage (28), and that oral supple-

ments with whey proteins, which contain high amounts of sulfur AAs, increase plasma GSH levels in patients with HIV(29). 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), upregulates synthesis of other antioxidants. Van den Akker et al. showed that AA administration to preterm infants results in increased albumin synthesis (30) and albumin also exerts antioxidant properties (31). 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 (32, 33). 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 after birth resulting in lower plasma cysteine concentrations on day 2 than on day 1, whereas erythrocytic glutathione concentrations had increased on day 2. Although plasma and erythrocytes represents 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. In addition, cysteine might be used for synthesis of important proteins like albumin.

AOPP concentrations typically increase significantly in the first week in preterm infants, both in hypoxic and non-hypoxic infants (22). In the present study, however, plasma AOPP concentrations were not yet increased on day 2, even though the maximum inspired oxygen fraction was significantly higher on day 2. 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. 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 (34). 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 (35). Regrettably, we could not obtain enough plasma for determining these markers, as a consequence of limited possibilities of blood withdrawal in these preterm infants.

The proportion of cesarean sections in the Day 1 group was significantly higher than that in the Day 2 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 (36-39).

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 cystine concentrations (13) decrease GSH breakdown to release free cysteine remains to be clarified.

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10 High dose cysteine does not stimulate glutathione synthesis in parenterally fed preterm infants

Frans WJ te Braake, Henk Schierbeek, Andras Vermes, Jan GM Huijmans, and Johannes B van Goudoever

Submitted

Abstract

Cysteine is thought to be the rate limiting substrate for glutathione synthesis, the major intracellular antioxidant. Our aim was to evaluate whether administration of additional cysteine is safe and stimulates glutathione synthesis in preterm infants' early life.

We conducted a prospective, randomized clinical trial in infants with a birth weight <1500 gram (n=20). They were randomly assigned to receive either a standard dose (45 mg/(kg·d)) or a high dose of cysteine (81 mg/(kg·d)). Intakes of other amino acids were similar, providing a total protein intake of 2.4 g/(kg·d) in both groups. Safety was confirmed by monitoring pH and base excess in the first 6 days of life. On postnatal day 2, we conducted a stable isotope study to determine glutathione concentrations and synthesis rates in erythrocytes.

Except for a lower base excess on the third day of life in the supplemented group ($p=0.03$), safety parameters were not clinically different between groups. Despite an 80% increase in cysteine intake, plasma cystine concentration did not increase. Glutathione concentrations and glutathione synthesis rates did not increase upon additional cysteine administration.

In conclusion, administration of a high dose of cysteine (81 mg/(kg·d)) to preterm infants appears clinically safe, but does not stimulate glutathione synthesis as compared to a lower dose (45 mg/(kg·d)).

Introduction

Cysteine, a nonessential amino acid (AA) synthesized de novo from methionine and serine, has been considered essential in preterm infants for the last decades. This notion was primarily based on experiments in human fetal tissues demonstrating lack of cystathionase activity, the enzyme catalyzing the final step in the cysteine synthesis pathway (1-3). In support of these observations, cysteine concentrations in preterm infants not receiving cysteine through their diet were found to be lower than those in term infants and the human fetus (4, 5).

Although recent studies of Riedijk et al. and Thomas et al. demonstrated adequate capacity of cysteine synthesis in enterally fed preterm infants (6-8), this might not hold true for preterm parenterally fed infants in the first postnatal days. Apart from the fact that the cysteine synthesizing pathway might not be fully active (9), demands may be increased directly after birth. Indeed, apart from being a substrate for protein synthesis, cysteine is considered to be the rate limiting substrate for glutathione (GSH) synthesis. GSH is required to prevent oxidative stress, which is strongly associated with a number of serious diseases observed in the neonatal period, such as bronchopulmonary dysplasia and periventricular leukomalacia (10). GSH is found in low concentrations in preterm infants deprived of exogenous AA administration (11). In these infants, concentrations of cysteine or its dimer cystine are particularly low (12, 13). As cysteine is feared to cause metabolic intolerance and to be unstable in parenteral solutions, most neonatal parenteral AA solutions contain only marginal amounts of cysteine or none at all. On the other hand, providing cysteine might decrease the need for methionine, which is associated with hepatotoxicity (14). In addition, it increases calcium and phosphorus solubility by lowering the pH of the AA solution (15).

Hypothesizing that a higher cysteine intake is metabolically safe and results in increased GSH synthesis rates, we conducted a randomized clinical trial in very low birth weight (VLBW) infants comparing effects of a standard (45 mg/(kg·d)) versus a high dose (81 mg/(kg·d)) of parenterally administered cysteine.

Methods

Design

The study was designed as a randomized clinical trial performed in the neonatal intensive care unit of the Erasmus MC – Sophia Children's Hospital, Rotterdam, the Netherlands between March 2007 and September 2007. The study was investigator initiated with no funding from industry. The protocol was approved by the Erasmus MC Medical Ethical Review Board and informed written parental consent was obtained prior to the study.

Patients

Subjects were inborn premature infants with a birth weight <1500 g who had an indwelling arterial catheter for clinical purposes, and were expected to be exclusively dependent on parenteral nutrition for the first 2 days of life. Besides glucose, all neonates received AAs directly following birth (2.4 g/(kg·d)) (Primene 10%, Baxter, Clintec Benelux N.V., Brussels, Belgium). Standard cysteine concentration of Primene is 1.89 g/L, thus providing a daily dose of 45 mg/kg (0.37 mmol/L) at an AA intake of 2.4 g/(kg·d). Directly after birth, infants were randomly assigned using a random-number table to receive either AAs including this standard amount of cysteine (CYS) or AAs including additional cysteine (CYS^+) (Hospira, Inc. Lake Forest, IL, USA, and SICOR Pharmaceuticals, Inc., Irvine, CA, USA) providing a total daily cysteine intake of 81 mg/kg (0.67 mmol/L). The additional cysteine was administered by the attending nurse. This was performed in a non-blinded fashion, since the study was not placebo-controlled. Intake of all other AAs did not differ between groups. Glucose and AAs were administered in separate solutions. Lipids and vitamins were not administered until after the stable isotope study on postnatal day 2 had been concluded. Exclusion criteria included erythrocyte transfusions within 12 hours prior to the study or during the study, known congenital abnormalities, chromosome defects, and metabolic, endocrine, renal, or hepatic disorders.

Study endpoints

Primary endpoint with respect to efficacy of cysteine administration was GSH concentration and synthesis rates in erythrocytes on postnatal day 2. Secondary endpoint was safety of cysteine administration, as confirmed by monitoring pH and base excess (BE) for the first six days of life. For all infants, we recorded birth weight, gestational age, birth weight Z-scores, and severity of illness at entry of the study by means of Apgar and CRIB scores (16). We also recorded requirements for base administration. Furthermore, we monitored use of supplemental oxygen and also documented antibiotics administration and incidence of sepsis, as sepsis is known to contribute to oxidative stress (17). We daily recorded actual nutritional intakes. We recorded plasma AA concentrations on the second day of life both as a safety and an efficacy parameter.

Tracer infusion protocol and sample collection

[1-^{13}C]Glycine (99% enriched, sterility and pyrogenicity tested) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and was dissolved in a 0.9% saline solution, filtered (0.2 µm) and sterilized by the hospital's pharmacy. The final product was tested for identity, content, sterility and pyrogenicity.

On postnatal day 2 neonates received a primed (40 μmol/kg) continuous (20 μmol/(kg·h)) infusion of [1-^{13}C]glycine for 6 hours. Blood samples (400 μL each) were drawn from an indwelling arterial catheter after 4, 5, and 6 hours and collected in EDTA containing microtainers to quantify erythrocyte free glycine enrichment, GSH-bound glycine enrichment and GSH concentration. Samples were immediately put on melting ice after centrifugation at 3500 x G for 10 min at 4°C. The plasma fraction was removed and stored separately for measurement of individual AA concentrations. The lower layer containing primarily erythrocytes was reconstituted to its original volume with ice-cold distilled water to disrupt cell membranes. The plasma and cell fractions were subsequently stored at -80°C until further analysis.

Glutathione and amino acid measurements

We measured GSH as total GSH, i.e. GSH + oxidized glutathione (GSSG). Enrichments of GSH-bound glycine and GSH concentrations were determined according to a recently developed technique, using an LC-Isolink interface (Thermo Electron, Bremen, Germany) coupled to a Delta XP isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany) (LC-IRMS) (18). This highly sensitive method requires only a small sample volume (200 μL whole blood), and does not require derivatization (18). Gas chromatography-combustion-isotope ratio mass spectrometry was used for analysis of 13C- isotopic enrichment of intraerythrocytic glycine, similar to an earlier developed method for measurement of the isotopic enrichment of threonine (19). Plasma concentrations of the direct GSH precursors glutamate, glycine, and cysteine (in vitro oxidized and measured as cystine), and indirect precursors glutamine, methionine and serine, as well as taurine, the product of cysteine catabolism, were determined with a Biochrom 30 amino acid analyzer using ninhydrin detection (Biochrom Ltd, Cambridge, England).

Calculations

The FSR_{GSH} was measured according to the product/precursor equation. It represents the fraction of the total intraerythrocytic GSH pool that is renewed per unit of time, and is expressed as %/d.

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

where E stands for enrichment expressed as mole percent excess (MPE). The nominator (product) of this equation represents the hourly increase of incorporated [1-^{13}C]glycine into GSH as calculated from the increase in enrichment between 4 and 6 hours of infu-

sion. The denominator (precursor) represents the intraerythrocytic free [1-^{13}C]glycine enrichment at isotopic steady state. A steady state plateau was defined as an insignificant change with time in intraerythrocytic enrichment. Subsequently, the intravascular absolute synthesis rate (ASR_{GSH}) was calculated by the following equation:

ASR_{GSH} (mg/(kg·d)) = FSR_{GSH}/100 x conc x 307 x ht x 0.075

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

Statistics

Statistical analyses were performed using SPSS version 15.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). Data are expressed as means ± SD or as medians (min – max). Primary outcome of the study was the glutathione fractional synthesis rate. Based on an abstract by Shew et al. (20), which describes a similar study, we calculated that with an α of 0.05, a power of 0.80 and a difference in FSR_{GSH} of 30%/d with an SD of 10%, group size needed to be at least three to detect a difference. We included 10 infants in each group in order to increase power.

Differences between groups were determined using independent t-tests or Mann-Whitney tests in case of normal or skewed distribution of the study groups, respectively. A P value of <0.05 was considered as statistically significant.

Results

We included twenty VLBW infants, equally distributed between the two groups. Twenty-seven infants were initially assessed for eligibility, of whom six infants were excluded due to not meeting inclusion criteria (n=2) or parental refusal (n=5). Group characteristics are shown in Table I. Stable isotope infusion was initiated at 30 ± 7 hours and 30 ± 6 hours postnatally in the CYS and CYS^+ group, respectively. As anticipated by study design, daily cysteine intakes were significantly higher in the CYS^+ group during the entire study period. Actual AA intakes as well as non protein calorie intakes for both groups are shown in Figure 1. Both total AA and non protein calorie intake were not different between groups at any day. None of the infants developed sepsis during the study. All infants were ventilated and therefore received prophylactic antibiotics according to our NICU's policy. Administration of these antibiotics was stopped in all infants after 48-72 hours since C-reactive protein concentrations were low and blood cultures were negative.

Table I. Clinical characteristics.

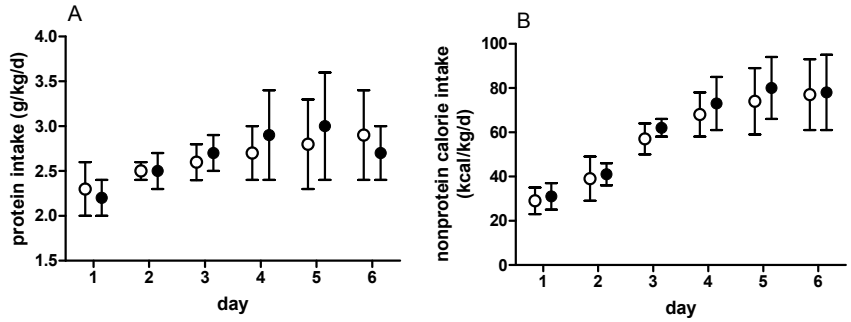
	CYS	CYS+
N (M:F)	10 (6:4)	10 (8:2)
birth weight (g)	978 ± 274	1006 ± 120
gestational age (wks)	28 ± 1 5/7	27 3/7 ± 1 2/7
birth weight Z-score (SD)	-1.1 ± 1.7	-0.3 ± 1.5
mode of delivery vaginal:cesarean section	5:5	3:7
Apgar score (5 min)	9 (4 – 9)	8 (6 – 10)
CRIB score	3 (1 – 10)	2 (1 – 5)

Values are expressed as means ± SD or medians (min – max) when appropriate.

There are no differences between groups.

Table II shows fractions of inspired oxygen in both groups until after the isotope study on day 2 (median (min – max)). No differences between groups were observed.

Figure 1. Protein intake (A) and non protein energy intake (B) in preterm infants receiving a parenteral cysteine intake of either 45 mg/(kg·d) (○) or 81 mg/(kg·d) (●). The sum of parenteral and enteral intake is shown. Values are expressed as means ± SD. There are no differences between groups.



Safety aspects

Figure 2 shows pH, BE, and bicarbonate infusion during the first 6 days of life. Besides a statistically significant difference in BE on the third postnatal day (p=0.03), pH or BE were not higher in the CYS^+ group. Infants in the CYS^+ group did, however, require more base administration on days 3 (p=0.048), 4 (p=0.002), and 5 (p=0.002).

Relevant plasma AA concentrations are shown in Table III**;** they did not differ between groups. In particular concentrations of cysteine, measured as its dimer cystine, were

Table II. Minimal and maximal fractions of inspired oxygen as from birth onwards until the end of stable isotope infusion.

	CYS	CYS+
Minimal FiO2 (%)	21 (21 – 27)	21 (21 – 22)
Maximal FiO2 (%)	33 (23 – 89)	31 (21 – 68)

Values are expressed as medians (min – max). No differences between groups were observed.

not higher in the CYS$^+$ group, despite an almost double intake. Also concentrations of taurine, a product of cysteine catabolism, were not significantly increased in the CYS$^+$ group.

Glutathione concentration and synthesis rates

All infants in both groups reached isotopic steady state after 4 hours of infusion, as represented by a non significant rise in intraerythrocytic [1-^{13}C]glycine enrichment (Figure 3). Mean precursor enrichments were 3.6 ± 0.6 and 3.3 ± 0.6 in the CYS and CYS$^+$ group, respectively. Erythrocyte GSH concentration was 1.83 ± 0.28 mmol/L in the CYS

Figure 2. pH (A), BE (B), and bicarbonate infusion (C) in preterm infants receiving a standard (○) or a high parenteral cysteine intake (●). Values are expressed as means ± SD (A and B) or means ± SEM (C). * Statistically significant difference.

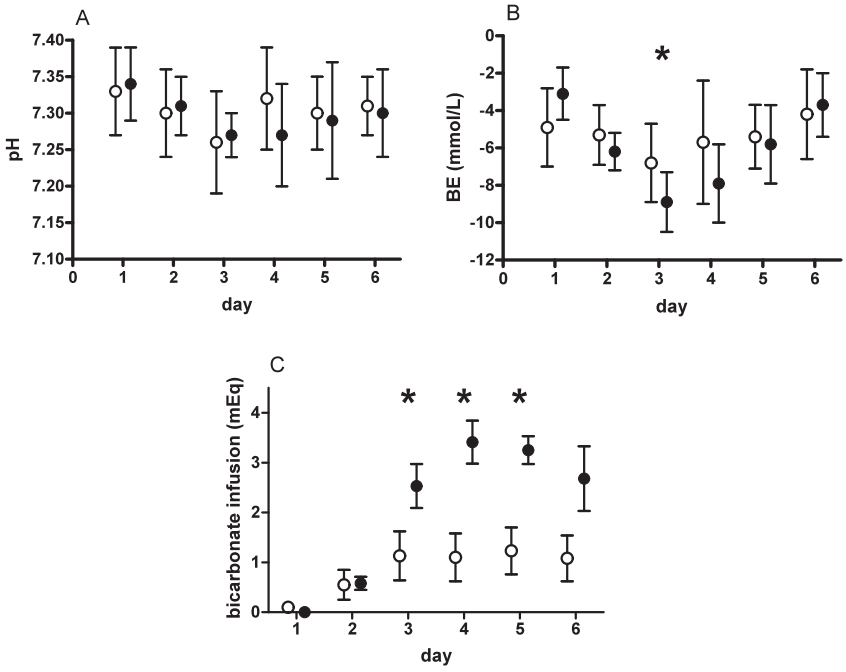


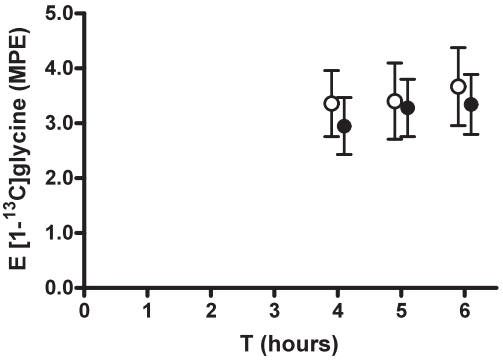
Table III. **Plasma amino acid concentrations.**

	CYS	CYS+
glutamate	57 ± 28	61 ± 27
cystine	32 ± 10	33 ± 14
glycine	325 ± 120	281 ± 106
glutamine	570 ± 190	516 ± 176
serine	179 ± 54	166 ± 37
methionine	45 ± 24	33 ± 11
taurine	73 ± 55	85 ± 54

Values are expressed as means ± SD. There are no significant differences between groups.

group and 2.02 ± 0.18 mmol/L in the CYS^+ group (p=0.10). FSR_{GSH} was identical: 48 ± 11 %/d and 48 ± 8 %/d in the CYS and CYS^+ group, respectively. ASR_{GSH} was 8.9 ± 2.2 mg/(kg·d) and 9.6 ± 2.1 mg/(kg·d) (p=0.49). Figure 4 shows individual data of each patient. We did not detect a significant gender specific difference in either plasma cystine concentrations or GSH kinetics.

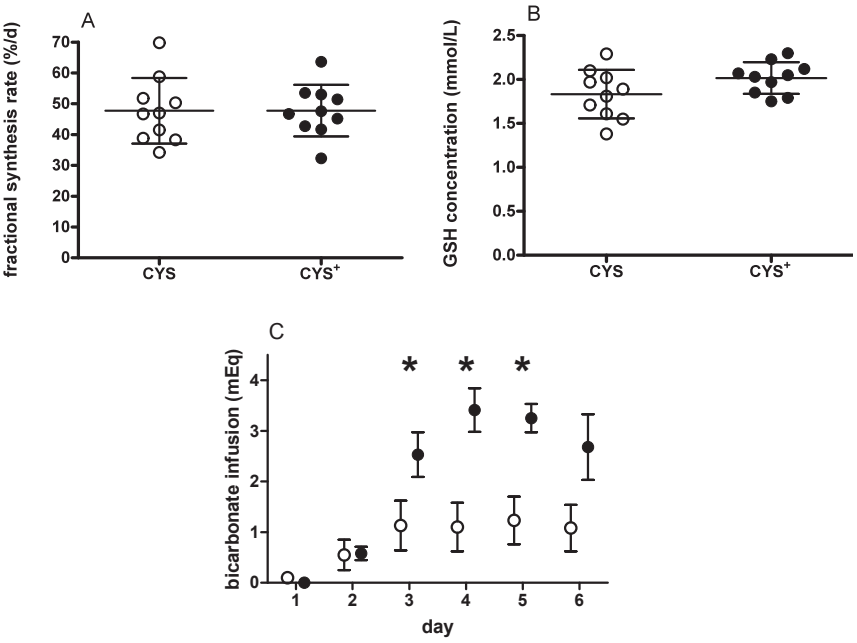
Figure 3. **Isotopic steady state was obtained in the standard dose (○) and the high dose group (●) after 4 hours of infusion, as represented by a non significant rise in intraerythrocytic [1-13C]glycine enrichment. Values are expressed as means ± SD.**



Discussion

As cysteine is feared to cause metabolic intolerance and to be unstable, parenteral AA solutions contain only low amounts of cysteine or none at all. Few products offer separate cysteine solutions to be added to the AA mixture just prior to administration. In this randomized clinical trial, we demonstrate that it appears safe to administer

Figure 4. GSH fractional synthesis rate (A), GSH concentration (B), and GSH absolute synthesis rate (C) in the standard dose (CYS) and high dose (CYS+) group. Individual values are shown. The horizontal lines represent means ± SD. No differences were observed between the groups.



cysteine at a dose of 81 mg/(kg·d), although infants in the high dose group did require more base administration on several days. In a previous trial we showed that GSH synthesis was stimulated upon administration of 2.4 g/(kg·d) including cysteine at a dose of 45 mg/(kg·d) (11). The higher dose of cysteine in this trial nevertheless does not further stimulate GSH synthesis as compared to the lower dose, nor does it increase plasma cystine concentrations. There are a number of explanations. First, the additional cysteine might conserve GSH by acting as an antioxidant itself, decreasing its consumption. This should, however, have resulted in either a higher GSH concentration or a lower synthesis rate while having similar concentrations. In addition, a higher cysteine dose might very well preserve GSH through inhibition of its breakdown, as one of the main functions of GSH is being a cysteine reservoir. Since GSH synthesis is feedback inhibited, GSH concentrations might be adequate already in the CYS group. In agreement, Cho et al. observed that feeding cystine above the supposed requirements did not increase GSH of any tissue in growing rats (21). Another explanation for the lack of increase in GSH synthesis rate might be that cysteine is used for other purposes. In the present study, the additional cysteine does not seem to be broken down to form taurine, as taurine concentrations hardly rose upon a higher cysteine dose. Besides for GSH synthesis, cysteine is likely to be rate limiting for a number of proteins as well and,

consequently, a higher cysteine availability might be preferentially incorporated into proteins such as albumin. Albumin has major antioxidant capacities apart from other functions and is rapidly upregulated upon AA administration in preterm infants directly following birth (22). In addition, cysteine is abundantly present in acute phase proteins (23). Using cysteine for other purposes than GSH would also explain why plasma cystine concentrations did not rise upon a higher cysteine dose.

Compromised bioavailability of cysteine in preterm infants could be another explanation why GSH synthesis rates did not increase. For example, when simultaneously delivered with glucose in a TPN bag, cysteine is known to form adducts (24). However, glucose and AAs are infused separately at our NICU. We infused cysteine as cysteine-HCl, which is more stable in solutions than cysteine. Infused cysteine is subsequently rapidly taken up by the neutral amino acid transport system ASC and might then be used for GSH synthesis (25, 26). On the other hand, plasma is much more oxidized than the intracellular environment and free cysteine might be auto-oxidized to cystine, which is transported intracellularly via another transporter, the xc-transport system. This carrier also transports glutamate, which competes with cystine.

Lavoie et al. demonstrated a maturity dependent fashion of cellular cysteine uptake in vitro (27). Apart from maturity, gender was found to influence cellular cysteine uptake. Still, the present study did not find any correlation between gender and erythrocyte GSH concentrations in, possibly reflecting a type II error.

To overcome stability issues, more stable cysteine analogues such as N-acetylcysteine (NAC) have been administered to improve GSH availability or clinical outcome in preterm infants. Although NAC has been shown to be very effective as GSH enhancing substrate in rodents as well as human children and adults (28-30), it did not have any effect on lung damage in preterm neonates (31, 32). In children and adults, NAC is rapidly taken up by cells and deacetylated subsequently, making it available for GSH synthesis. The apparent relative inability of the preterm infant to deacetylate was considered after the observation that 53% of the intake of NAC and 38% of the intake of N-acetyl tyrosine (NAT) was excreted unmetabolized in urine of one-week-old preterm infants (33). Moreover, plasma concentrations of NAC and NAT were higher than those of cysteine and tyrosine. So far there are no data on long term safety aspects of these acetylated products.

A limiting factor for GSH synthesis, other than cysteine, might be another explanation for the present findings. Since the enzymes necessary for GSH synthesis are present already during midgestation and are readily upregulated in case of increased GSH requirements, these are unlikely to be limiting GSH synthesis (34, 35). With respect to availability of other substrates: glycine has been found to be essential in preterm infants in a number of studies. Van Lingen et al., using [15N]glycine, found that hardly any added tracer was detectable in urinary urea, particularly in small for gestational

age infants (12). In addition, Jackson et al. measured urinary 5-L-oxoproline in preterm and term infants. Higher concentrations of 5-L-oxoproline are associated with decreased availability of glycine for GSH synthesis. The authors found a significant inverse linear correlation between the excretion of 5-L-oxoproline and length of gestation or birth weight (36). Indeed, glycine might be limiting under particular circumstances. Still, plasma concentrations of both glycine and glutamate, which are used as cysteine in equimolar amounts to synthesize GSH, are much higher than cystine concentrations in the present as well as other studies.

Because of ethical considerations, we did not include a group receiving no cysteine at all. The importance of cysteine availability per se in relation to GSH has been confirmed earlier in healthy adults in which turnover of GSH accounts for approximately half the cysteine flux in the post absorptive state (37). Two studies in preterm infants, presented only in abstract form, demonstrated a significant rise either in GSH fractional synthesis rates (erythrocytes) or concentrations (whole blood) upon amino acid administration containing cysteine as compared to no cysteine (20, 38). Rats fed a low protein diet (3%) were unable to increase GSH production upon administration of 98% oxygen, in contrast to rats fed a 25% protein diet. When the 3% diet was supplemented with cysteine, lung GSH concentrations increased during oxygen exposure. However, supplementation of the 25% protein diet with cysteine did not further protect these animals (39).

Conclusions

Cysteine administered at a dose of 81 mg/(kg·d) appears to be safe, but does not increase GSH synthesis rates as compared to a dose of 45 mg/(kg·d). Further research should be directed towards defining the requirements of cysteine in the parenterally fed preterm neonate in the direct postnatal phase.

Acknowledgements

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11 Glutathione metabolism and oxidative stress in a NICU population

Frans WJ te Braake, Karien de Groof, Henk Schierbeek, Mariangela Longini, Giuseppe Buonocore, Dick Tibboel, and Johannes B van Goudoever

Abstract

Critically ill infants requiring aggressive ventilatory support are subjected to a high load of reactive oxygen species (ROS) and may experience serious oxidative stress and therefore have increased antioxidant consumption. Glutathione (GSH) is the major intracellular antioxidant but might not function optimally in the direct neonatal phase.

We performed an observational study including infants suffering from perinatal asphyxia, infants requiring extra corporeal membrane oxygenation (ECMO), and relatively healthy, partially enterally fed preterm infants. We measured plasma concentrations of advanced oxidized protein products (AOPP) and dityrosine, both markers of oxidative stress. In addition, using stable isotopes ([1-^{13}C]glycine) we determined GSH fractional and absolute synthesis rates in erythrocytes.

Eleven asphyxiated infants, eight infants on ECMO, and ten preterm infants were included in the first week of life. Both AOPP and dityrosine concentrations were present in significant amounts in all groups. As compared to relatively healthy preterm infants, GSH concentrations and synthesis rates are not higher in asphyxiated infant, but GSH absolute synthesis rates were higher in infants on ECMO. This was primarily due to the larger pool size in infants on ECMO.

In conclusion, both asphyxiated infants, infants requiring ECMO and preterm infants experience major oxidative stress as reflected by the presence of markers of oxidative stress. GSH absolute synthesis rate is markedly higher in infants receiving ECMO therapy.

Introduction

Critically ill neonates requiring intensive care are often subjected to aggressive treatment modalities, including ventilatory support with high fractions of inspired oxygen. Hyperoxia, but also hypoxia and reperfusion are known to generate significant amounts of reactive oxygen species (ROS), which may cause irreversible cellular damage if not counterbalanced by adequate antioxidant availability. Indeed, these infants are prone to develop oxidative stress, which may contribute to the risk of morbidity and mortality (1, 2).

Glutathione (GSH), the major intracellular antioxidant, built up from glutamate, cysteine, and glycine, is synthesized de novo within almost all cells (3). As we have recently shown, its synthesis is readily upregulated following amino acid (AA) administration in preterm infants receiving hyperoxic treatment (4). A number of clinical conditions and treatment strategies involving increased ROS production are likely to increase GSH demands. Here, we report on oxidative stress and antioxidant defense in i) asphyxiated infants receiving conventional ventilatory support and ii) infants treated with extra corporeal membrane oxygenation (ECMO) for various reasons, such as congenital diaphragmatic hernia or meconium aspiration syndrome, and iii) relatively healthy preterm infants.

Perinatal asphyxia

Incidence rates of perinatal asphyxia vary dependent on definitions being used, but may occur in 1.8 to 6.9 per 1,000 live borns (5). ROS, which are believed to play a role in the development of hypoxic ischemic encephalopathy in asphyxia (6), are formed during the hypoxic event, and in the reperfusion phase directly following hypoxia (7), as well as during resuscitation with high concentrations of oxygen (8). In the postnatal course, the AI is often in need of mechanical ventilation with supraphysiologic oxygen concentrations, which can cause further intracellular ROS formation.

Treatment with extra corporeal membrane oxygenation (ECMO)

ECMO is a highly invasive procedure that uses an artificial lung to take over the work of the lungs with regard to gas exchange, and depending on the mode (veno-venous or arterial-venous), also the heart. ECMO is used most often in newborns and young children and may be continued up to several weeks. Indications for ECMO are very strict and the number of infants and children eligible for ECMO treatment is small. The purpose is to support or replace temporarily failing lungs by providing oxygen and removing carbon dioxide waste products so the lungs can rest and recover. Infants needing ECMO may

include those with meconium aspiration syndrome, persistent pulmonary hypertension, and congenital diaphragmatic hernia. One of the characteristics of ECMO treatment, and in view of this study of particular interest, are the dramatic fluctuations of partial oxygen pressure (pO2). A high pO2 is associated with low GSH availability (9).

Augmenting GSH availability by increasing its production rate might be effective to remedy oxidative stress. We therefore conducted an observational study to measure synthesis rates of GSH in erythrocytes of asphyxiated infants and ECMO treated infants. We compared these infants with a group of relatively healthy, anabolic preterm infants, since it is ethically not justified to determine GSH kinetics in healthy term infants, due to the required venous access and blood sampling. To quantify oxidative stress, we measured advanced oxidized protein products (AOPP) and dityrosine, both markers of protein oxidation (10, 11). We hypothesized that asphyxiated infants and infants requiring ECMO experience high amounts of oxidative stress which is compensated for by a high GSH synthesis shortly after birth.

Methods

Patients

Asphyxiated infants, infants requiring ECMO treatment, and relatively healthy preterm infants, admitted to the neonatal intensive care unit and the children's intensive care unit of the Erasmus MC - Sophia Children's Hospital in Rotterdam, respectively, were enrolled in the study. Infants were born between September 2005 and June 2007. Written and oral parental informed consent was obtained prior to study. The study was approved by the Erasmus MC Medical Ethical Review Board. The following inclusion criteria were applied for the group of asphyxiated infants: gestational age > 35 weeks; umbilical cord pH <7.10; 5-minute Apgar score ≤ 6 and clinical signs of asphyxia (Sarnat stage II or III). Exclusion criteria were known congenital abnormalities, chromosome defects, hemolytic disorders, endocrine, renal or hepatic disorders or transfused with erythrocytes during or within 12 hours prior to the study. During and prior to the study, asphyxiated infants received parenteral nutrition and, if tolerated, minimal enteral feeding.

Inclusion criteria for ECMO treatment were failure to respond to conventional medical support including high frequency ventilation, surfactant therapy, inhaled nitrogen oxide (20 ppm), vasopressors and hydrocortisone. Infants were eligible for ECMO when the oxygenation index was higher than 40. Infants on ECMO received both parenteral and enteral nutrition.

Inclusion criteria for preterm infants were birth weight < 1500 g and an indwelling arterial catheter. These infants received both parenteral and enteral nutrition as well. Exclusion criteria were similar to the group of asphyxiated infants.

At entry of the study, we recorded birth weight, gestational age, and Apgar scores for all infants. For the asphyxiated infants we also recorded umbilical cord pH, BE, and postnatal lactate levels. We also recorded plasma amino acid concentrations, and protein intake.

Tracer infusion protocol

[1-^{13}C] glycine (99% enriched, sterility and pyrogenicity tested) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and was dissolved with a 0.9% saline solution by the hospital's pharmacy after it was again tested for identity, sterility and pyrogenicity.

All infants received a primed (40 µmol/kg) continuous (20 µmol/kg/h) infusion of [1-^{13}C]glycine for 6 hours. Blood samples (400 µL each) were collected from an indwelling arterial catheter in microtainers containing EDTA after 4, 5 and 6 hours of infusion. Samples were immediately put on melting ice after which they were centrifuged for 10 minutes (3500 x *g*) at 5ºC. 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 to disrupt cell membranes. The plasma fraction and cell fraction were subsequently stored at -80ºC until further analysis.

For studying GSH kinetics, we used a recently developed technique described previously, based on liquid chromatography coupled to isotope ratio mass spectrometry (LC-C-IRMS) (Thermo Electron, Bremen, Germany) (12). This highly sensitive method requires only a very small sample volume, whereas no derivatization is required. Erythrocyte [1-^{13}C]glycine was considered the precursor for GSH synthesis and was measured according to our earlier developed method on the derivatization of threonine (13, 14).

Plasma concentrations of direct GSH precursors glutamate, glycine, and cysteine (measured as cystine), and indirect precursors methionine and serine, were determined with a Biochrom 30 amino acid analyzer, using ninhydrin detection (Biochrom Ltd, Cambridge, England). GSH synthesis is depicted in Figure 1.

Calculations

The fractional synthesis rate (FSR_{GSH}) represents the fraction of the total intraerythrocytic GSH pool that is renewed per unit of time, and is expressed as %/d. It was measured according to the precursor-product equation:

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

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 with time in intraerythrocytic enrichment. Subsequently, the intravascular absolute synthesis rate (ASR_{GSH}) was calculated by the following equation:

ASR_{GSH} (mg/(kg·d) = FSR_{GSH}/100 x conc x 307x ht x 0.070 *(asphyxiated and preterm infants)*

or

ASR_{GSH} (mg/(kg·d) = FSR_{GSH}/100 x conc x 307x ht x 0.210 (*infants on ECMO*)

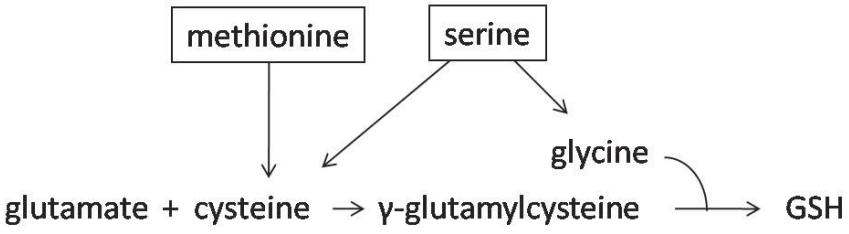
where conc is concentration in mmol/L packed erythrocytes, 307 is the molecular weight of GSH, ht is hematocrit, and 0.070 and 0.210 are the estimated circulating volumes expressed as L/kg, in asphyxiated and preterm infants, and infants on ECMO, respectively.

Plasma marker of oxidative stress

We measured the advanced oxidation protein products (AOPP) in plasma by the spectrophotometric assay described by Witko-Sarsat et al. (10). 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.

Dityrosine has been recognized as an oxidative stress product of pathological response to disease or other environmental stress (11, 15). Its concentration was measured according to the method described by Abdelrahum et al., based on liquid-liquid extraction, reversed-phase chromatography and fluorescence detection (16).

Figure 1. GSH synthesis pathway.



Statistics

Statistical analyses were performed using SPSS v14.0 (SPSS Inc, Chicago, IL, USA). Data are expressed as means ± SD or as medians (min – max).

Results

Eleven asphyxiated infants, eight infants on ECMO, and ten preterm infants were included. Clinical characteristics of both groups as well as the group of preterm infants can be found in Table I.

Asphyxiated infants had a mean umbilical cord pH of 6.89 ± 0.16, an umbilical cord base deficit of 21 ± 7, and a lactate concentration of 8.3 ± 5.5 on postnatal day one. All asphyxiated infants were resuscitated with 100% oxygen, and subsequently ventilated with additional oxygen for at least one day.

Infants in the ECMO group were receiving arterial-venous ECMO treatment for the following reasons: three infants had a congenital diaphragmatic hernia, three infants suffered from meconium aspiration syndrome, one infant had a congenital cystic adenomatoid malformation of the lung, and one infant had an omphalocele. Asphyxiated infants were studied on postnatal day three, and preterm infants on postnatal day six. Infants on ECMO were studied at an age of 6 ± 3 days, while ECMO was initiated at day 5 ± 3 days of life.

The total protein intake of the day of study in the asphyxiated infants was 1.8 ± 0.6 g/(kg·d) and in the ECMO group 2.3 ± 0.5 g/(kg·d). In the group of preterm infants protein intake was 2.7 ± 0.3 g/(kg·d).

Oxidative stress markers are shown in Figure 2. AOPP levels in asphyxiated infants, infants on ECMO, and preterm infants were 247 ± 83, 178 ± 47, and 293 ± 76 µmol/L, respectively. Dityrosine concentrations were 3.6 ± 5.0 ng/mL in asphyxiated infants, 15.5 ± 10.2 ng/mL in infants on ECMO and 9.6 ± 3.5 ng/mL.

Figure 3 shows enrichment of the precursor, free intraerythrocytic [1-^{13}C]glycine, between four and six hours of infusion. In all groups, a plateau was reached after four hours of infusion, which was defined as no significant increase in enrichment.

Figure 4 shows erythrocyte GSH concentrations, FSR_{GSH} and ASR_{GSH}.

FSR_{GSH} was 52 ± 12 %/d in asphyxiated infants, 65 ± 17 %/d in infants on ECMO, and 49 ± 11 %/d in preterm infants. Erythrocyte GSH concentration was 1.9 ± 0.3 mmol/L in asphyxiated infants, 2.0 ± 0.3 mmol/L in infants on ECMO, and 1.6 ± 0.4 mmol/L in preterm infants.

Due to the larger pool size in infants in the ECMO group, ASR_{GSH} are clearly higher in the ECMO group. Mean values are 9 ± 2 mg/(kg·d) in asphyxiated infants, 23 ± 4 mg/(kg·d) in infants on ECMO, and 6.0 ± 1.4 mg/(kg·d) in preterm infants.

Table II shows plasma concentrations of all AAs directly or indirectly involved in GSH synthesis in asphyxiated infants, infants on ECMO, preterm infants as well as a reference group of healthy term breast-fed infants (17).

Discussion

Seriously ill infants such as included in this study require intensive care treatment, which is primarily focused on vital functions, such as cardiorespiratory support. Oxida-

Table I. Clinical characteristics of asphyxiated infants (Asphyxia), infants on ECMO (ECMO) and preterm infants. Data are expressed as mean ± SD. 1Data adapted from Te Braake et al. 2008.

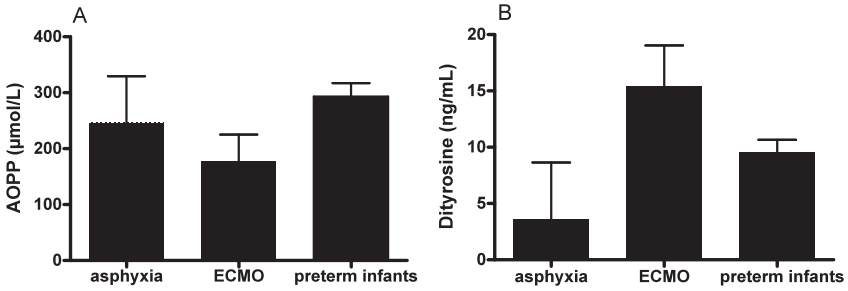
	Asphyxia	ECMO	Preterm infants1
N (male: female)	11 (7:4)	8 (4:4)	10 (8:2)
Gestational age (wks)	39 2/7 ± 2	39 1/7 ± 1 5/7	26 4/7 ± 1 1/7
Birth weight (g)	3458 ± 612	3170 ± 337	820 ± 134
Apgar score (5')	3 ± 2	6 ± 2	7 ± 3

tive stress is not anticipated for by any specific form of treatment, although it may have profound effect on outcome. In this study we used AOPP and dityrosine to reflect protein oxidation. Because amino acids, peptides and proteins are vulnerable to attacks by ROS, these attacks can result in amino acid aggregation, cross linking and fragmentation (18). AOPP and dityrosine concentrations thus reflect protein oxidation following oxidative stress and can be considered as reliable markers of oxidative stress. Previous studies on AOPP in the neonate showed elevated AOPP levels in hypoxic premature neonates at birth and at the 7th day of life (19, 20). From this study, it follows that both asphyxiated infants, infants requiring ECMO treatment and preterm infants experience oxidative stress, evidenced by presence of AOPP, comparable to results earlier obtained in preterm infants (20), and dityrosine.

Oxidative stress in asphyxiated infants is in agreement with earlier studies on lipid peroxidation in the neonatal period, showing elevated levels of non protein bound iron, a sensitive indicator of oxidative stress, and lipid peroxidation in asphyxiated infants compared to healthy neonates (21, 22). Moreover, urinary excretion of lipid peroxidation products may be used to predict death in perinatal asphyxia (23).

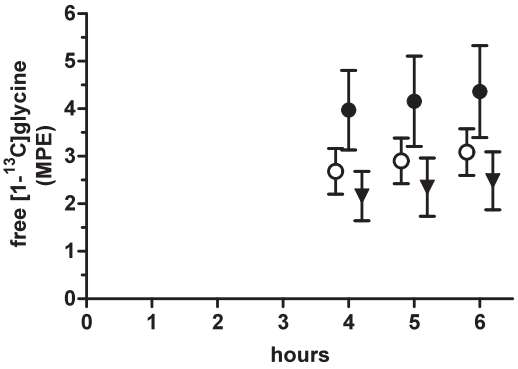
Despite presence of oxidative stress, this is not compensated for by an increased FS-R_{GSH} or ASR_{GSH}. Although asphyxiated infants received the lowest protein intake, plasma concentrations of GSH substrates were all within reference ranges. The relatively low

Figure 2. **AOPP (A) and dityrosine (B) concentrations. The grey bar shows data obtained in relatively healthy anabolic preterm infants. Bars present mean ± SD.**



ASR_{GSH} might be explained by the fact that the measurements were performed on postnatal day 3, whereas the hypoxic event had occurred in the perinatal period. Although it might be argued that the most dramatic impact on GSH demands is expected to

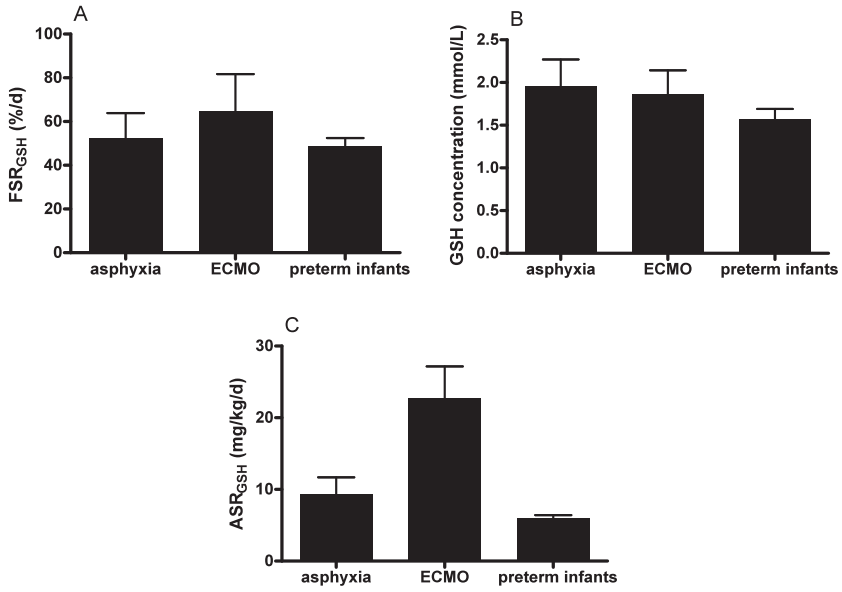
Figure 3. **Precursor enrichment (MPE). Values are depicted as mean ± SD. ●asphyxiated infants; ○infants on ECMO; ▼ preterm infants.**



occur in the direct postnatal phase, ethical constraints impeded measurements in this critical period. On the other hand, the asphyxiated neonates are ventilated with high concentrations of oxygen for hours to days, which earlier resulted in elevated levels of oxidized GSH on postnatal day 3 which remained present up to 4 wks of age (8, 24). As a response to prolonged ROS production, FSR_{GSH} and ASR_{GSH} would be expected to be increased for days and therefore measurable on postnatal day 3. Another explanation for the seemingly low GSH synthesis rates might be hepatic dysfunction as a sequella of asphyxia. The GSH synthesis apparatus is primarily produced by organs such as the liver and kidney which are known to be very sensitive to asphyxia inflicted damage.

At time of our study, 100% oxygen was administered to resuscitate asphyxiated infants. Nowadays, the use of 100% oxygen is no longer the standard of care in the

Figure 4. **FSR_{GSH} (A), erythrocyte GSH concentration (B), and ASR_{GSH} (C). Data are expressed as mean ± SD.**



resuscitation of the newborn. Using room air instead of 100% of oxygen was found to be safe and resulted in a better outcome of asphyxiated infants (25, 26). Currently, we don't know the effects of a lower fraction of inspired oxygen on oxidative stress and GSH kinetics, but future studies will be performed to investigate this issue.

Table II. **Plasma concentrations (µmol/L) of amino acids involved in GSH synthesis in term healthy infants. Data are expressed as mean ± SD [1]Adapted from Wu et al. 1986.**

	Asphyxiated infants (n=11)	ECMO (n=8)	Preterm infants (n=10)	Term healthy infants[1]
Glutamate	105 ± 39	108 ± 71	53 ± 13	76 – 551
Cystine	40 ± 14	48 ± 33	23 ± 11	33 – 55
Glycine	380 ± 79	376 ± 21	318 ± 135	66 – 432
Serine	203 ± 59	188 ± 79	171 ± 45	79 – 227
Methionine	36 ± 6	41 ± 19	22 ± 6	21 – 55

GSH is synthesized by glutamate, glycine and cysteine. The latter is considered the limiting substrate for GSH synthesis. Although ECMO enables the lung to rest and recover, it is a highly invasive procedure and results in a profoundly hypermetabolic and catabolic state (27, 28). However, nutrition is not considered of primary importance in

these infants resulting in low cysteine availability (29). In fact, to minimize burden to the gastrointestinal tract, some of these sick infants receive parenteral nutrition as their primary source of nutrition during the acute phase of their illness. This might negatively impact on antioxidant defense and augment oxidative stress. In healthy lambs, it was found that treatment with ECMO resulted in a significant drop in antioxidant enzymes, such as superoxide dismutase and glutathione reductase (30).

Our data show that the intravascular ASR_{GSH} in infants on ECMO is higher than in both asphyxiated infants and preterm infants. Although infants on ECMO showed to have the highest FSR_{GSH}, their significantly higher circulating volume is predominantly responsible for their ASR_{GSH}. However, there are some limitations with respect to these data. First, the ECMO system contains a large amount of donor blood and most infants frequently require transfusions. Although the in vivo half life of GSH in erythrocytes under conditions of oxidative stress is unknown, it is conceivable to have influenced our data. However, blood transfusions are inherent to ECMO treatment, and we performed our measurements at least 12 hours after the last transfusion. In addition, by measuring the FSR_{GSH}, we measured real time GSH synthesis.

Second, the significantly higher ASR_{GSH} might not seem surprisingly considered the higher circulating volume. On the other hand, erythrocytes have shown to be powerful donors of antioxidant defense, including GSH, to other tissues (31-33). In addition, the ECMO system is not metabolically active.

Because antioxidants work synergistically, the decline of one oxidant can be prevented by another antioxidant. Therefore the GSH concentration and kinetics alone do not allow for a total antioxidant activity in the neonate. Although GSH is the main intracellular antioxidant which has concentrations in the millimolar range, by only measuring GSH kinetics, we demonstrated a limited aspect of total antioxidant status in relation to oxidative stress.

In conclusion, in this study, we showed that both anabolic preterm infants, term asphyxiated infants as well as term infants on ECMO experience considerable oxidative stress. In asphyxiated infants, this was not compensated for by an increased GSH synthesis rate, which is not due to a lack of substrate availability. It might be inherent to a relatively deficient GSH synthesizing apparatus.

Infants on ECMO show significant GSH synthesis rates, although interpreting these values requires caution due to the high circulating volume.

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12 General discussion

Introduction

Historically, innovations in the early postnatal management of preterm infants have primarily been targeted to immediate life threatening issues, such as respiratory and circulatory support and prevention of infectious disease.

Nutrition is increasingly being recognized as a crucial part of treatment strategies for preterm infants. However, nutritional requirements and tolerance of preterm infants seem to differ markedly from term newborns who are capable of digesting breast milk which provides principally all essential nutrients to meet their specific demands. At present we have not determined these specific nutritional requirements of preterm infants, both in terms of quality and quantity. Moreover, in defining their needs, we are troubled by issues such as fluid restrictions, intolerance to enteral feeding, and heterogeneity of the population.

This thesis focuses on amino acids in particular, investigating their effects on different biochemical indices of neonatal wellbeing, i.e. protein synthesis and antioxidant defense.

In the following paragraphs the major findings and their clinical implications will be discussed in the context of relevant literature. Subsequently, some methodological considerations will be dealt with and future research purposes will be highlighted.

Major Findings

PART I – amino acid administration and protein metabolism

The first part of this thesis has dealt with safety aspects and anabolic effects of early AA administration in preterm infants. Most of the body growth and development of preterm infants, such as discussed in this thesis, occurs outside of the uterus. Indeed, when the umbilical cord is cut, nutrient delivery abruptly ceases, while organogenesis is still incomplete at preterm birth. This necessitates rapid restoration of nutrient supply with the goal of minimizing interruption of growth and development.

In the absence of parenteral nutrition and not being able to receive enteral feedings in the direct postnatal period, an infant is dependent on its own protein stores for obligatory protein catabolism, costing approximately 1.0 g/(kg·d) or 1 to 2 % of its endogenous protein stores each day when receiving glucose only(1). An age-matched fetus will still be accreting protein at a rate of approximately 1.5 g/(kg·d). The latter growth rate should actually be aimed at in the feeding of preterm infants, but is often not achieved(2, 3). As a result, many infants born appropriate for gestational age will leave the hospital small for gestational age(4).

In the first part of this thesis we described early nutritional strategies to promote anabolism in the first days of life. We showed that administering AAs (2.4 g/(kg·d)) directly following birth is safe and converts a negative nitrogen balance into a positive one (**chapter 2**). In addition, using stable isotopes, we demonstrated this anabolic state to be caused by increased protein synthesis rather than decreased proteolysis (**chapter 3**). Several studies on AA kinetics have shown a marked difference in protein metabolism between preterm infants and term infants. There is a negative correlation between gestational age and protein loss, resulting in a doubling of the protein losses in ELBW infants as compared to term infants(5). During protein administration, term infants(6) and adults(7) respond by decreasing proteolysis, whereas fetuses(8) and preterm infants(9-13) increase protein synthesis, rather than suppressing breakdown.

In **chapter 3**, we found that the energy required for protein synthesis was not derived from increased glucose oxidation, but seemed partly derived from increased AA oxidation. Increased AA oxidation is likely to be the cause of the higher blood urea nitrogen (BUN) levels we found in infants receiving AAs as compared to infants receiving glucose only.

Amino acid administration directly following birth is safe and converts a catabolic into an anabolic state. Energy for protein synthesis is not derived from increased glucose oxidation.

An ideal nutritional strategy provides for amounts of AAs and energy that satisfy the needs for both growth and metabolism. There is ongoing controversy whether AAs should be regarded as metabolic fuel or not. Their main function lies in protein synthesis, but it remains unsolved to what extent it is physiological for AAs to be oxidized for energy generation. Reasons for AAs to be oxidized include energy generation in general, and second, to avoid accumulation in case one or more AAs are present *pro rata* more abundantly than those needed for the composition of proteins to be synthesized.

In the ovine fetus, the umbilical supply of AAs highly exceeds the amount deposited for tissue growth. Oxidation contributes 25 to 50% of fetal AA uptake(14, 15). To what extent this also holds true for the human fetus is largely unknown, since human fetal studies are restricted by technical and ethical concerns.

Studies have demonstrated that AA administration combined with as little as 30 non-protein kcal/(kg·d) can turn the nitrogen balance from negative into zero or even positive(16-18). Advanced feeding strategies and improved neonatal AA solutions have changed suggested AA intake related to minimal energy intake from approximately 1.0 g AAs per 30 kcal non-protein energy to 1.0 g AAs per 15 kcal nonprotein energy. As this is not an ideal proportion of energy for optimal protein synthesis, a considerable amount of the AAs will be oxidized. An intake of 25 to 40 kcal of nonprotein energy per gram of

protein will enhance optimal protein deposition, though this is not feasible with glucose alone in early preterm life at larger protein intakes(19). However, the effect of increasing energy intakes on protein deposition will be greatest below 50-60 kcal/(kg·d), above which the beneficial effect of extra energy ceases and the amount of administered AAs itself will have a higher correlation with anabolism(20). Besides its anabolic effects, AA administration can increase maximal non-protein caloric intake. This can be partially attributed to enhanced insulin secretion following AA administration (notably arginine and leucine)(21). Many studies indeed found lower glucose concentrations during AA administration(12, 18, 22).

Early lipid administration, with its high caloric content of 9 kcal/g, might be beneficial in delivering calories for the cost of protein synthesis. Furthermore, the infant is dependent on essential fatty acids for its brain maturation. The fetus receives only a small amount of lipids at the beginning of the third trimester, which raises the question whether the very preterm infant is suited to metabolize lipids in large amounts. As compared to AAs, data on early administration of lipids are scarce. Supposed metabolic intolerance (increased risks of sepsis, displacement of bilirubin from albumin, and pulmonary complications) and an association with development of BPD have led to their delayed introduction, often beyond the first 24h postnatally. A recent review from the Cochrane Collaboration found no statistically significant effects of early introduction of lipids on short term nutritional or other clinical outcomes. Neither benefits nor adverse effects were demonstrated in the studies reviewed(23). However, no AAs had been administered in the studies included in the review. Concomitant administration of AAs could theoretically have enhanced lipid utilization. Indeed, Ibrahim et al. administered both AAs and lipids to a small group of preterm infants directly from birth onwards, which resulted in an anabolic state without increased BUN levels, suggesting adequate use of lipids as energy source(22).

Our present knowledge of feeding the preterm infant is based on research over the last decade in relatively healthy preterm infants. Today, however, we treat ELBW infants, who have higher nutritional requirements than VLBW infants, which are even more difficult to meet as a consequence of the clinical problems involved. To underline this assumption, in a group of 69 ELBW infants energy and protein deficits were found to be inversely related to birth weight(24).

In **chapter 3** we demonstrated stimulatory effects of AAs on whole body protein synthesis, as calculated from leucine incorporation into body protein. Studying whole body metabolism has limited value, however, since it only provides information on the average of all metabolic processes in the body rather than organ specific changes. Therefore, we studied whether administration of AAs directly following birth also stimulates organ specific protein synthesis as compared to glucose only in VLBW infants. We studied albumin synthesis, which can be quantified by measuring the incorporation rate

of [1-^{13}C]leucine into plasma albumin (**chapter 4**). Our data show increased albumin concentrations and synthesis rates following AA administration as compared to infants receiving glucose only. Seeing that albumin has important functions, among which serving as an antioxidant and binding bilirubin and free fatty acids, stimulating albumin synthesis might positively influence outcome.

Early nutrition and clinical outcome

The clinical trials presented in Part I have investigated immediate effects of AA administration on protein metabolism (**chapter 2, 3, 4**). Short-term outcome of early nutrition has found to be beneficial. Whether improvements in the postnatal phase are extended throughout the neonatal period and thereafter is still unclear. On the other hand, improving nutritional care in the direct postnatal phase might contribute to decreased infant and early childhood morbidity. Outcome criteria of early nutrition can be based upon different aspects, such as intrauterine growth charts(25) or growth charts obtained from premature infants(3, 26, 27), incidence of specific disease, hospital stay, etc. Although relatively little is known about medium or long-term outcome parameters involving early nutrition, it is known that impaired growth due to suboptimal nutrient intake in preterm infants has both adverse consequences during hospitalization and after discharge. Short-term consequences might include high infectious disease vulnerability arising from suboptimal immune defense, free radical mediated damage caused by impaired antioxidant production, and increased requirement for ventilatory support, partially due to muscle weakness(28).

Some studies found favorable effects of early and/or high AA supplementation in the neonatal period on the incidence of BPD(28, 29) and retinopathy of prematurity (ROP) (28) whereas others did not(30, 31). In most studies anthropometric measurements had improved at discharge(30-33) whereas this effect seemed to be disappeared at 18 months of age(30). An explanation is that preterm infants are likely to have periods of growth delays during hospitalization related to the medical or nutritional consequences of their illnesses and some former preterm infants will therefore remain small throughout life.

Not until the 1990s scientists began to show more interest in effects of early malnutrition on neurodevelopment(34, 35). A study of Latal-Hajnal et al. in 219 VLBW infants found favorable effects of a rapid postnatal weight-gain on neurodevelopment, as assessed by Bayley Scales of Infant Development(34). In contrast, no differences were found in a larger study (including over 1000 ELBW infants) conducted by Poindexter et al. at 18 months corrected age(30). However, in this study infants who received ≥3 g/(kg·d) of parenteral AAs within the first 5 days of life were considered "early", which in fact might not be considered early according to current opinion.

In **chapter 5** we presented data describing anthropometric data and neurodevelopmental outcome in our cohort of infants described in **chapter 2**. The intervention group received AAs (2.4 g/(kg·d)) as from birth onwards, whereas the control group received glucose only during the first two days of life. Although a trend towards less handicaps was observed in the intervention group, these follow-up data do not show any statistically significant differences between groups. This on the one hand reconfirms safety, and on the other hand would suggest no clinical benefit for the preterm infant. Recently, concerns have been raised towards safety of early AA administration(36). Therefore, this follow up study was conducted primarily to exclude adverse long-term effects. Whether early AA administration has significant clinical benefits for early childhood and later life, needs to be studied in larger trials specifically designed and thus powered to answer these questions.

Increased susceptibility for later disease is another nutrition related long-term effect which has gained a lot of interest. Of particular interest is the Barker Hypothesis. This theory states that by adapting to an environment with limited supply of nutrients (metabolic imprinting), such as early neonatal malnutrition, the fetus preferably reserves its nutrients for essential organs such as the brain. As a consequence, 'nonessential' organs such as the pancreas and kidney may become deprived. Reduced cell growth in these organs during critical periods of malnutrition results in susceptibility to cardiovascular disease, diabetes mellitus or renal insufficiency(37).

Early amino acid administration is safe with regard to neurodevelopmental outcome at two years of age. Large randomized clinical trials are needed to study whether early amino acid administration improves outcome in early childhood and later life.

PART II – amino acid administration and antioxidant defense

The second part of this thesis has dealt with nutritional aspects of oxidative stress and glutathione (GSH) metabolism. Essentially, there are two different means of preventing harm caused by oxidative stress: the first is to prevent excess formation of reactive oxygen species (ROS), i.e. beyond physiological levels. However, this seems not a very realistic goal, since, as stated in **chapter 1**, ROS are generated during many events and in many pathways, such as hyperoxia, ischemia-reperfusion, and activation of phagocytes. The second means of preventing toxicity by oxidative stress is to enhance antioxidant defense systems, which can intercept ROS or repair damage. Early nutrition is a key-factor in promoting antioxidant defense and has been the main subject throughout this part of the thesis. Although GSH is the main intracellular antioxidant, it is important to realize that total antioxidant defense is the result of the interplay between

other intracellular and extracellular antioxidants, such as vitamin C and vitamin E. GSH synthesis is primarily dependent on presence of its synthesizing enzymes, activity of AA transport mechanisms across cellular membranes, and availability of substrates. In **chapter 7** we demonstrated that birth weight and Z-score for birth weight are inversely correlated to GSH fractional synthesis rate (FSR_{GSH}), thereby confirming the GSH synthesizing apparatus to be active. Our data agree with results obtained from earlier studies demonstrating presence of these enzymes in leukocytes obtained from tracheal aspirates and cord blood and in autopsy samples of fetal liver, lung, and kidney(38, 39). In addition, even during starvation, GSH synthetic enzymes appear to be maintained for readiness of GSH synthesis upon substrate availability(40).

Uptake of GSH substrates is another requirement for GSH synthesis. Lavoie et al. determined L-[^{35}S]cysteine uptake capacity in leukocytes of cord blood and tracheal aspirates of infants of varying gestational ages(41). It was found that the maturity of female but not male newborns was positively correlated with cysteine uptake and from this it was concluded that cysteine uptake might be compromised in preterm infants. However, we did find active GSH synthesis in erythrocytes of preterm infants which increased dramatically upon AA administration. This can only be achieved by uptake of substrates, including cysteine. In addition, our data in **chapter 3 and 4**, showing increased whole body protein synthesis and albumin synthesis upon AA administration, suggest intact cysteine uptake in other cells besides erythrocytes. Also, Shew et al. demonstrated increased GSH synthesis rates upon administration of cysteine(42). However, these data were only presented in abstract form. Whether there is a *relatively* compromised uptake of cysteine or the results of Lavoie et al. represent an *in vitro* artifact remains undetermined.

The final requirement for GSH synthesis is availability of substrate. Collectively, the results in this thesis suggest this to be the most important determinant in GSH availability in preterm infants in the immediate postnatal phase. This will be further discussed below.

Glutathione availability in the preterm infant

In 1958, Szeinberg et al. were among the first to report on GSH metabolism in newborn infants(43). They concluded that GSH concentration in cord blood and blood obtained in the early neonatal phase was significantly higher than in adult blood. Still, term newborns experienced a "transient decreased resistance of GSH to oxidative agents" during the first hours of life, which could be ameliorated by providing glucose or inosine to the infant. The mechanism by which the glucose and inosine exerted their protective effect remained unclear.

In 1984, Lestas et al. compared concentrations of GSH, GSH reductase, GSH peroxidase, and GSH synthesizing enzymes glutamate cysteine ligase and GSH synthetase between fetal blood (17-24 wks gestation) and neonatal and adult blood(44). In parallel with Szeinberg et al, they found higher GSH concentrations in fetuses (cord blood) compared to adults. From these studies, it seemed that there would be a certain preparation for transition to the extrauterine environment. It was speculated that this would anticipate for oxidative stress arising from increased oxygen exposure and other oxidant stressors such as inflammation, UV radiation, etc. Frank et al. first demonstrated this concept in vivo studying antioxidant enzymes in developing rabbit lung(45). It was found that during the final days in utero, superoxide dismutase and catalase activities increased by approximately 110% whereas lung GSH peroxidase activity increased by approximately 200%. More recently, this phenomenon was demonstrated in human infants as well, where term labor was associated with increased erythrocyte GSH concentrations whereas preterm labor was not(46).

Nowadays we are able to treat increasingly preterm infants. Improved methodology enables measurements of GSH concentrations in smaller sample sizes as required in these infants. Early studies of Szeinburg et al., suggesting high GSH concentrations in the near term human fetus, contrasts with values obtained in newborn preterm infants, which are usually found to be low. In most studies involving human neonates, concentrations of GSH and/or its derived enzymes are determined in whole blood, plasma, erythrocytes, bronchoalveolar lavage fluid, or occasionally tissue samples obtained during surgical procedures or autopsy. Although caution should be exercised when comparing those studies due to the different analytical techniques being used, the majority of studies report a drop in GSH concentration in preterm infants during the first days of life or a low GSH concentration as compared to term infants, older children or adults(47-50). When comparing these studies to our study, a marked difference in nutritional intakes between preterm infants is observed. In the studies reporting low GSH concentrations in preterm infants as compared to term infants, AAs were either not provided at all, or did not include any cysteine, which is generally present in low concentrations in plasma of preterm infants.

From our studies in human infants, it follows that the compromised GSH availability in erythrocytes of preterm infants is primarily due to nutritional deprivation which can easily be converted into concentrations which seem to be adequate upon more aggressive nutritional support. Similar, Chessex et al. fed neonatal guinea pigs either intravenous glucose or glucose and AAs. When challenged with oxygen therapy, only the AA group increased liver GSH twofold(51).

In **chapter 8**, we demonstrated that FSR_{GSH} on the second day of life was not higher in the group receiving AAs as from birth onwards as compared to infants receiving glucose only, suggesting feedback inhibition of GSH synthesis following decreased GSH

consumption. This is supported by the fact that we did not find a further increase in GSH synthesis upon administering a higher dose of cysteine, generally considered as the rate limiting substrate for GSH synthesis (**chapter 10**).

Decreased glutathione concentration in blood from preterm infants is due to nutritional deprivation and can be stimulated by early amino acid administration. In addition, in response to early amino acid administration, glutathione consumption seems to be decreased.

As stated above, GSH concentrations in fetal blood are higher than in blood of children and adults(44). However, in **chapter 9** we found significantly lower GSH concentrations a few hours after birth as compared to the second day of life. We speculate that GSH concentrations fall shortly after birth. This rapid drop is in agreement with the observation that GSH concentrations decrease in the first hours following oxidative stress in neonatal piglets and young rats, which was followed by a nearly normal or sometimes increased level 24 hours later, suggesting a compensatory effect(52, 53). Yet, this effect only occurred if substrate availability was sufficient. Also in preterm infants, there was a drop GSH content during the first 24h of life(54). Unfortunately, in this study no information was provided on nutritional intakes.

Our data demonstrate a rapid depletion of glutathione postnatally in preterm infants. This relative deficiency is reverted by early administration of amino acids.

The FSR_{GSH} did not significantly change upon changes in nutritional intake. As compared to sick term infants (**chapter 11**) or healthy adults (**chapter 8**), FSR_{GSH} was consistently low. However, a number of studies have shown GSSG recycling to be increased in preterm infants, which would decrease the need for *de novo* synthesis(55, 56).

Cysteine. A limiting substrate?

Cysteine is a non essential AA. Its carbon skeleton is derived from serine. The sulfur component is derived from methionine, which is converted to homocysteine through the intermediate S-adenosylmethionine. Homocysteine and serine are then combined to form cystathionine, which is subsequently converted into cysteine by the enzyme cystathionase. Cysteine is involved in many different pathways, of which protein and GSH synthesis are quantitatively most important. In healthy adults in the postprandial state, half of the cysteine flux could be accounted for by the turnover of GSH(57). Indeed, GSH and cysteine metabolism are strongly interdependent: cysteine is unstable as a

free AA and is therefore stored in GSH, serving as an 'on demand' cysteine reservoir. Alternatively, GSH requires cysteine to carry out its antioxidant properties. GSH thus both stores and consumes cysteine.

In general, cysteine is considered the rate limiting substrate for GSH synthesis(58, 59). Indeed, its concentrations in several tissues is significantly lower than glutamate and glycine concentrations that are both used in equimolar amounts in the GSH synthesis process. Although glycine demands are probably increased in preterm infants, being involved in the synthesis of many other AAs, utilization of glycine for erythrocyte GSH synthesis represented less than 2% of the plasma glycine flux(53). However, besides cysteine availability per se, its synthesis seems more dependent on the properties of glutamate cysteine ligase (GCL), the rate limiting enzyme in GSH synthesis. In humans, the *Km* values of GCL for glutamate and cysteine are 1.8 and 0.2 mM, respectively(60). The intracellular concentration of glutamate is significantly higher than the *Km* value of GCL for glutamate, whereas the intracellular concentration of cysteine almost equals the corresponding *Km* value of GCL. From this theoretical point of view, it follows that cysteine is indeed the rate limiting substrate for GSH synthesis.

In term infants with respiratory distress receiving ventilation with high fractions of inspired oxygen, plasma cysteine concentrations were significantly lower than fasted infants not treated with additional oxygen or healthy fed infants(61). However, it remains unclear whether cysteine is actually essential to these infants, especially during increased demands, such as in the case of oxidative stress. A number of authoritative papers published in the 1970s found cysteine to be essential in preterm infants due to absence or low activity of cystathionase in the fetal liver(62, 63). Recently, however, Riedijk et al. performed a series of studies determining cysteine requirements in enterally fed preterm infants. Using sophisticated stable isotope methods, they found cysteine not to be essential in preterm infants with a mean gestational age as low as 28 weeks(64, 65). Whether this equally applies to parenterally fed preterm infants in the immediate postnatal phase remains unclear. Miller et al. administered [U-^{13}C]glucose to parenterally fed infants and did not detect any enrichment in cysteine. From this, they concluded that cysteine synthesis is decreased in parenterally fed preterm infants. On the other hand, during the study infants received AAs already containing cysteine at a dose of nearly 90 mg/(kg·d)(66). It can therefore not be concluded that cysteine synthesis was compromised in these infants.

Animal data has shown GSH levels to be increased upon cysteine supplementation in TPN solutions(67, 68). Data on effects of cysteine administration on GSH metabolism in human neonates are scarce. In fact, as far as we know, only two abstracts have been presented(42, 69), reporting increased erythrocyte GSH synthesis and concentrations, respectively. Zlotkin et al. performed a series of studies on the effects of cysteine supplementation on nitrogen and sulfur balances and weight gain(70, 71). A

control group received an AA solution without cysteine; the intervention group received cysteine-HCl at dose of 77 mg/(kg·d), which is almost similar to the amount infants in our high dose group received (**chapter 10**). They found no differences between groups and from these papers it was concluded that cysteine was either not essential or not bioavailable to the preterm infant. However, mean gestational age of these infants was >34 weeks, whereas mean study-age was postnatal day 14. In addition, hepatic cystathionase activity in preterm infants was found to reach adult levels after 9 days of life(72), suggesting that these infants were already able to synthesize cysteine. Extrapolating these data to preterm infants included in our studies seems, therefore, incorrect. During increased cysteine requirements, e.g. sepsis and oxidative stress, the transsulfuration pathway may not be able to provide for this increased demand. Another reason why it is likely that GSH consumption is increased during sepsis is the liberation of cysteine which can subsequently be incorporated into acute phase proteins, which contain a high content of cysteine.

In **chapter 10** we studied the effects of a high cysteine dose on erythrocyte GSH kinetics as compared to a standard dose. We did not find any difference in either GSH concentration or synthesis rates. Surprisingly, also plasma cystine concentrations did not rise. Infants receiving the high dose cysteine did require increased base administration, indicating that the higher cysteine dose was indeed administered.

Because of its instability in solutions, bioavailability of cysteine has been questioned. Moreover, cysteine is said to be auto-oxidized in plasma to cystine, liberating reactive species(73). Though perhaps true, it would be expected to increase protein oxidation markers such as advanced oxidized protein products (AOPP). In **chapter 10** we found no difference in AOPP concentrations in infants receiving either the high or the standard cysteine dose. Still, its stability in solutions is limited requiring its addition to an AA solution just prior to administration. Therefore, a number of alternative sources of cysteine have been proposed. N-acetyl cysteine (NAC) is more stable than cysteine-HCl and seemed promising. Indeed, NAC has been successfully applied in increasing GSH availability in nutritionally deprived children and adults with respiratory distress(59, 74). Very recently, it has shown to reduce cerebral oxidative stress in asphyxiated newborn piglets(75). However, NAC seems to have limited bioavailability to the preterm infant. Its administration resulted in increased plasma NAC and not cysteine concentrations. In addition, over 50% of NAC was excreted unmetabolized in urine, suggesting that preterm infants have decreased capacity to deacetylate NAC(76). Oxothiazolidine carboxylate, a 5-oxoproline analogue, is metabolized by 5-oxoprolinase and converted to cysteine. Alike NAC, it has been shown to increase GSH concentrations in adults with respiratory distress(74). Lipoic acid, used as a dietary supplement, reduces cystine to cysteine which is more easily transported intracellularly and thus enhances GSH

synthesis(77). Both oxothiazolidine carboxylate and lipoic acid, however, have yet not been tested for use in preterm infants.

Current ESPEN/ESPGHAN guidelines on pediatric parenteral nutrition (2005) advice the administration of cysteine to preterm infants. They state that an intake of 0.35 mmol (42 mg/(kg·d)) to 0.46 mmol (56 mg/(kg·d)) would result in appropriate plasma concentrations. Based on our results, we cannot recommend increasing cysteine intake above 45 mg/(kg·d) as far as GSH synthesis concerns. Although we did not observe adverse metabolic effects, plasma cystine concentrations were not increased, which might reflect its use for other purposes such as protein synthesis, or its catabolism to sulfate.

High dose cysteine (81 mg/(kg·d)) appears safe but does not increase glutathione concentration or synthesis rate in erythrocytes of preterm infants as compared to a lower dose (45 mg/(kg·d)).

Clinical outcome

Antioxidants and oxidative stress represent a scientific area consisting of believers and nonbelievers. Optimizing antioxidant availability or preventing oxidative stress is expected to improve outcome from a theoretical point of view. Unfortunately, the majority of clinical trials failed to do so. Since pathophysiology of oxidative stress related morbidity is likely to be multifactorial, large numbers of infants are required to make useful inferences. In addition, there are many antioxidants and most of them work synergistically, forming an integrated balanced system of great complexity.

A large Scandinavian trial investigating the effects of NAC supplementation on incidence of bronchopulmonary dysplasia (BPD) did not show any reduction(78), although this might be due to the earlier mentioned compromised bioavailability of NAC in preterm infants(76). Davis et al. administered intratracheal superoxide dismutase or placebo to 302 preterm infants. No difference was found in incidence of BPD(79). Route of administration might be an important factor in determining success of antioxidant interventional studies. Cooke et al. found that intratracheal administration of GSH in ventilated preterm infants resulted in significantly higher levels of GSH in bronchoalveolar lavage fluid, whereas lipid peroxidation was reduced(80). This was, however, only a small study including 14 infants, so that it remains to be determined whether alternatives for systemic administration have therapeutic benefits. Also, timing of intervention might be an important determinant of its success. Indeed, the pathophysiological base for some oxidative stress associated diseases is set very early after birth(81). Intervening with an antioxidant or its precursor might incorrectly be concluded as not efficacious only because it was initiated too late.

Most clinical trials on antioxidant supplementation fail to show positive effects on clinical outcome.

Nevertheless, low antioxidant availability has been associated with poor outcome. In a study in 144 infants on plasma antioxidant concentrations in relation to incidence of BPD and mortality, Silvers et al. found low plasma antioxidant activity at birth to be an independent risk factor for mortality(82). In infants developing BPD, it was found that elevations in plasma oxidative stress markers were more pronounced in the early days of life, indicating the need for early intervention(83, 84).

Facts and Fiction of Oxidative Stress

Oxidative stress has been defined as "an imbalance between pro-oxidants and anti-oxidants in favor of the former, leading to potential damage. Indicators of oxidative stress include damaged DNA bases, protein oxidation products, and lipid peroxidation products"(85). Oxidative stress has been described in a large number of different pathological states and diseases, including protein deprivation, atherosclerosis, rheumatoid arthritis, diabetes mellitus, Parkinson's disease, and many others(86-89).

A certain amount of oxidative stress markers, however, are formed independently of oxidative stress, as is the case with carbonyls. Comparing concentrations of different markers in the same individuals can, therefore, lead to contradictory results as was observed by Mocatta et al. who found higher MDA concentrations in plasma of preterm neonates as compared to term neonates in the perinatal period, whereas carbonyl concentrations were lower(90). This example illustrates the importance of being cautious while interpreting presence of markers of oxidative stress.

According to its definition, oxidative stress is a pathological phenomenon. However, a number of recent publications report oxidative stress in seemingly physiological events such as term uncomplicated birth. As compared to children or adults, increased levels of isoprostanes were observed in healthy term neonates during the first three months of life. In addition, activity of superoxide dismutase and catalase increased during the same period, after which it decreased(91).

In another study, plasma MDA levels were found to be more than doubled in breast-fed infants as opposed to infants fed cow's milk(92). This was attributed to the presence of polyunsaturated fatty acids (PUFAs) in breast milk. Much controversy exists concerning the role of PUFAs as promoter of oxidative stress(93, 94). PUFAs double bonds are susceptible to free radical mediated lipid peroxidation. The resulting peroxyl radicals initiate a chain reaction, which can be halted by vitamin E.

Collectively, whereas we have seen that absence of nutrition will lead to depletion of antioxidants, its presence is likewise a potential source of oxidative stress.

Although parenteral AA solutions enhance GSH synthesis as shown in this thesis, TPN can be a source of free radicals(95). They generally contain riboflavins, which have oxidizing properties when exposed to light by means of promoting hydrogen peroxide production(96). It must be stressed, however, that these (per)oxidative effects of certain macro- and micronutrients are unlikely to have adverse clinical consequences, let alone that it would imply to be reticent about early nutrition. It does advocate a critical appraisal of composition of neonatal nutrition. The paradoxical effects of nutrition, might, nevertheless, explain the 'physiological' oxidative stress observed in healthy infants(91).

Caution should be exercised when interpreting levels of oxidative stress markers.

Halliwell has recently put forward a concept which might explain this 'physiological' oxidative stress. Since maintaining excess antioxidant defenses has a high energy cost, it could be energetically 'cheaper' to repair or replace damaged biomolecules(97, 98). In addition, a rise in oxidative stress markers might result from failure to degrade or repair biomolecules, rather than implying oxidative stress *per se*. In **chapter 7**, we found a rise in both AOPP and dityrosine concentrations in plasma of preterm infants towards postnatal day 6. Although irreversibly damaged proteins, such as AOPPs are likely to be rapidly degraded by proteases, we do not know their half life. It is probably safe, however, to conclude that oxidative stress is ongoing. In **chapter 8**, we found no difference in AOPP or dityrosine concentrations in plasma of preterm infants administered glucose only for the first two days of life or infants who received AAs as from birth onwards. Still, erythrocyte GSH synthesis rates increased significantly. We discussed a number of explanations, such as plasma and erythrocytes being different compartments.

Another issue raising controversy is mode of delivery. In our studies, mode of delivery did not affect either markers of oxidative stress or GSH kinetics. On the other hand, we did not perform measurements in cord blood. A number of studies have found a beneficial effect of vaginal delivery over cesarean section in terms of oxidative stress(99, 100); other studies concluded the opposite(101, 102), whereas some did not find a difference at all(103, 104). Infants born by cesarean section are on average more likely to experience distress while in utero. This could be a problem in studies in which this confounding factor is not accounted for. In a recent study, it was found that distressed fetuses delivered by emergency cesarean exhibited higher MDA concentrations in amniotic fluid and cord blood than non distressed fetuses delivered by elective cesarean section(105).

Tissue specific measurements

Although erythrocytes clearly have shown to be a target for ROS in preterm infants(106, 107), we did not obtain information on GSH kinetics in other tissues. This may have consequences for the interpretation and generalizability of our results. Deneke et al. performed an in vitro study exposing calf endothelial cells to an hyperoxic challenge. Using stable isotopes, they found that this was associated with an enhanced precursor AA uptake(108). However, when repeating this experiment in human erythrocytes, no stimulation of GSH synthesis was observed(109). Malmezat et al. found that in food restricted septic rats, both GSH concentrations and synthesis rates were significantly higher than in well-fed control rats in liver, spleen, muscle, lung, heart and large intestine(110). From these data, the authors concluded that the inflammatory challenge overcame the impact of food restriction. However, in the same study, GSH synthesis was lower in blood and small intestine. In contrast to this study, hepatic GSH synthesis in rats increased during inflammation only when adequate sulfur AAs were consumed before the inflammatory challenge(52).

In a study in humans, measuring GSH kinetics in whole blood, lower GSH concentrations and synthesis rates were found in malnourished septic children as compared to non-septic children. Although having essentially acquired the same data as Malmezat et al.(110), this study did not provide data on organ level GSH kinetics, which might had been increased in parallel Malmezat's findings. Indeed, GSH concentrations and synthesis rates in blood might decrease as to provide substrates for organ specific GSH synthesis during increased requirements, such as sepsis. On the other hand, apart from being an indicator for oxidative stress, erythrocytes might be able to provide antioxidant defense to other tissues as well, such as the lung, either by providing intracellular antioxidants(111) or by directly taking up ROS(112). Recently, active GSH export towards plasma has been demonstrated. This suggests a role for erythrocytes as an active GSH donor to other tissues and the extracellular GSH pool, a role which was previously thought to be performed by the liver only. This study, however, failed to elucidate the mechanism by which the GSH was exported, since no GSH transport systems have been identified yet. It was shown earlier that erythrocytes may actively donate antioxidants to other tissues(113). Barbaro et al. found GSH correlations in plasma and mononuclear cells to correspond to liver GSH concentrations, suggesting that in humans blood GSH may be a sensitive indicator of tissue concentrations(114).

Although erythrocyte glutathione concentration might not reflect glutathione status in all tissues, its concentration is decreased during systemic oxidative stress.

Methodological Considerations

GSH is receiving ongoing scientific interest. Annually, over 4,000 new articles are published. More than half of these studies is carried out in animals; some of them involve *in vitro* research; many different cell types are studied; age of subjects varies a lot. As a result, making general inferences about GSH often comes down to comparing apples to oranges. For example, GSH and its associated enzymes have shown to vary significantly among different animal species, including humans(115). Another study found GSH levels to be subjected to diurnal variation(116). Also, concentrations of plasma and erythrocyte GSH in the same subjects seem to rise and fall independently. However, these concentrations differ a thousand-fold, thereby increasing the likelihood of miscalculation of the plasma GSH concentration(117).

GSH is a central component in the process of eliminating ROS. However, in the absence of GSH-containing enzymes to catalyze the many different detoxification reactions, it cannot properly function as an antioxidant. In our series of studies, we measured total GSH only, without its derived enzymes. The main reason is the limited sample availability: kinetic measurements require multiple sampling. In addition, there is a large number of GSH dependent enzymes, including GSH peroxidases and GSH-S-transferases, and they are all equally important. The presence of reduced GSH, however, is a prerequisite for synthesis of all its derived enzymes(118).

In our study, we measured GSH concentrations in erythrocytes, whereas markers of oxidative stress were determined in plasma. In part, the rationale for this was limited sample availability in preterm infants. Although recent data suggest active GSH export from erythrocytes to plasma(113), they remain separate compartments, which might explain our results indicating ongoing oxidative stress in plasma despite increased erythrocyte GSH synthesis (**chapter 7 and 8**).

GSH concentrations are much higher than GSSG concentrations both in the physiological state but also during oxidative stress. Therefore, a minimum of artifactual GSH oxidation during sample treatment can lead to an overestimation of 10- to 100-fold(119). Since GSH is very susceptible to artifactual oxidation ex vivo, its measured concentration is highly dependent on methodology and sample treatment. This has resulted in a wide range of reported concentrations for both GSH and GSSG(119). Measurements of antioxidants, such as GSH, should therefore be standardized in order to compare results between different studies(120).

The heterogeneity of study design and applied methods requires caution when interpreting and comparing data.

We believe it is justified to be very skeptic towards measurements of GSSG concentration. A number of authors claim to find statistically significant correlations between GSSG concentrations and prematurity, fractions of inspired oxygen, etc. However, most of these studies included only a small number of infants, or present graphs showing correlations which are, in my opinion, questionable(47, 121, 122).

Future Perspectives

As in all research, many issues involving nutritional aspects of treatment of the preterm infant remain to be elucidated. Throughout this chapter we have discussed some of these questions. In this paragraph we will recapitulate our suggestions for future research.

With respect to early parenteral nutrition, safety and short-term efficacy of early AA administration should no longer be subject of debate. Focus should be directed towards long-term neurodevelopmental outcome. In addition, the maximal AA intake as well as the effects of early lipid administration as an energy source for protein synthesis need to be defined. The role of the composition of lipid infusions and how this is related to oxidative stress should be further investigated.

Whether cysteine is a limiting substrate for GSH synthesis in preterm infants remains to be determined. Although we did not find any increase in GSH synthesis rates upon doubling cysteine intake, we cannot be sure that cysteine-HCl has adequate bioavailability to the preterm infant. In addition, we quantified synthesis in erythrocytes. GSH synthesis might be increased in other tissues, such as the liver or the lungs.

A lot of controversies remain on appropriate use of oxygen in neonatal medicine. Especially with respect to resuscitation of the preterm neonate, its role should be further elicited. In term asphyxiated infants, there is an extensive body of literature on providing room air during resuscitation instead of additional or even pure oxygen. In particular Saugstad and Vento have performed a lot of research demonstrating increased oxidative stress in infants resuscitated with 100% oxygen as compared to room air. Strikingly, mortality decreases upon administering less oxygen. In addition, Apgar scores and clinical outcome, such as time to first cry and developing a sustained pattern of breathing, improve using room air. Also, resuscitation with 100% oxygen resulted in prolonged presence of markers oxidative stress even up to 4 weeks of age(123). Also, throughout the neonatal period, a restrictive approach towards use of oxygen seems advisable(124-127). However, data with regard to preterm infants are still inconclusive, in particular with respect to resuscitation policy(128). Another study found incidence of BPD to be almost doubled after resuscitation with 100% oxygen on the first day of life, BPD being defined as oxygen dependence on the 36th week postmenstrual age. Because it took place in two different centers which might applied different oxygenation manage-

ment style, results may have been blurred to some extent(129). Spector et al. reported on an association between oxygen administration for 3 minutes or longer and childhood cancer(130). This association has yet not to be proven causal though. Currently, large, multicenter, blinded randomized trials are being conducted to determine optimal oxygen saturation levels for preterm infants. As yet, no data have been published. In extending our current research design, GSH kinetics should be determined in preterm infants resuscitated with low versus high fractions of inspired oxygen.

Besides oxygen, the role of sepsis and its impact on GSH requirements should be further elicited. The development of sepsis during the first weeks of life is an important cause of morbidity and mortality of premature infants(131). Infants who develop sepsis have a significantly prolonged hospital stay and are more likely to die than those who do not develop sepsis. Previous research has shown that sepsis leads to an increased generation of ROS through different pathways and subsequent oxidative stress(132). ROS are generated as part of the physiologic response during sepsis, with the aim of killing pathogens(133-135). However, this response can be overstimulated, causing a shift in redox balance. The resulting oxidative stress causes tissue damage and could play a role in morbidity and mortality. Especially premature infants are at risk, because they have both compromised antioxidant defenses as well as increased ROS production as a result of additional oxygen requirements. To date, little is known about the specific interplay of oxidative stress and sepsis in premature infants.

Defining the optimal moment to intervene for prevention of oxidative stress needs further study. GSH levels seem to fall rapidly upon preterm birth whereas markers of protein oxidation and lipid peroxidation in early life have been positively correlated with adverse outcome, such as BPD(83, 84). Effects of administering antioxidants antenatally to pregnant mothers should be investigated. In mice, administration of NAC to infected pregnant mothers diminished oxidative stress, increased hepatic GSH levels and reduced the rate of preterm birth(136). In another study, pretreatment with vitamin C protected against fetal death in pregnant mice who were daily injected with lipopolysaccharide (LPS), an oxidant. Postnatal treatment with vitamin C of LPS-treated mice had only little effect on intrauterine fetal death(137).

The majority of clinical trials has investigated effects of a single antioxidant(78, 79). This is required to investigate applicability of a specific antioxidant as a potential therapeutic drug. Unfortunately, such studies ignore the fact that most antioxidants interact and require presence of another antioxidant to carry out their function, such as vitamin C and E. Some elements serve as a co-factor for other substances to function as an antioxidant, such as selenium, which is required to synthesize GSH peroxidase. However, providing selenium to preterm infants while GSH availability is compromised is less likely to increase GSH peroxidase availability.

We speculate that a cocktail of antioxidants is required to exert significant health benefits. There are some difficulties though. As stated in chapter 1, low levels of ROS are physiologic and serve important roles in cell signaling, gene expression, and cell-mediated cytotoxicity of pathogens(138). In addition, a number of antioxidants have pro-oxidant properties beyond certain concentrations, such as vitamin C and E. Although these are crucial antioxidants not made by the human body, caution should be exerted to prevent overdosing. In this context, providing adequately AAs will enable the infant to synthesize protein based antioxidants *ad libitum*, such as GSH and its derived enzymes, whose synthesis is feedback inhibited.

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13 Summary

Summary

Nutrition is increasingly being recognized as an important factor in the treatment of preterm infants. In this dissertation we present a number of studies on the effects of early parenteral amino acid (AA) administration on protein kinetics (Part I) and antioxidant defense (Part II).

In **Chapter 1** we provide a general introduction to early nutrition in preterm infants. Preterm infants are mostly dependent on parenteral nutrition during the first weeks of life. Unfortunately, the use of first generation AA solutions showed significant metabolic side-effects. Although present day solutions have been improved, many neonatologists are still reluctant to administer AAs in (a substantial amount) during early life. As a result, infants become in a catabolic state, slowing down growth as a whole as well as synthesis of important proteins, such as albumin. This is likely to negatively influence their short-term and possibly long-term outcome. In this thesis, we present studies on the effects of early AA administration in preterm infants on protein metabolism and antioxidant defense as indices of neonatal wellbeing.

Part I - early nutrition & protein metabolism

Chapter 2 describes a clinical trial investigating safety and efficacy aspects of parenteral AA administration directly following birth as compared to glucose only in a large group of preterm infants with a birth weight <1500 gram. We used a number of biochemical indices, such as pH and base excess, to confirm metabolic safety. Apart from higher blood urea nitrogen (BUN) levels in the group receiving AAs, we did not find metabolic disturbances. In our opinion, the higher BUN level, however, reflects increased AA oxidation rather than intolerance. Nitrogen balance turned positive upon AA administration, whereas the control group receiving glucose only had a negative nitrogen balance, meaning they were in a catabolic state. Besides promoting anabolism, AA administration resulted in higher plasma concentrations of (conditionally) essential AAs, which were more within reference ranges as compared to infants receiving glucose only. Thus, early AA administration seems safe and results in anabolism.

In **Chapter 3**, we studied the mechanism by which anabolism in response to exogenous AA administration is obtained in preterm infants in the direct postnatal phase. Using stable isotope techniques, we found that protein synthesis is stimulated, even during very low energy intakes. However, we did not find a concomitant decrease in proteolysis. Anabolism in adults and healthy term infants, unlike in preterm infants and ovine fetuses, is predominantly achieved by suppression of proteolysis instead of

protein synthesis. Possibly, a new balance between protein breakdown and synthesis is developing during early life, explaining this observed discrepancy.

We also measured glucose oxidation rate, which we expected to increase as a result of the higher energy demands necessary for protein synthesis. However, glucose oxidation did not rise during AA administration, suggesting that the increased energy demands are not made up for by glucose utilization.

Whereas we showed increased protein kinetics on a whole body level in chapter 3, in **Chapter 4** we determined synthesis rates of albumin, the main plasma protein. We found that early AA administration resulted both in increased fractional synthesis rates as well as in increased concentrations. This finding might have important implications in view of the vital roles of albumin, among which serving as an antioxidant and binding bilirubin and free fatty acids.

In the earlier chapters we demonstrated beneficial effects of AA administration in the direct postnatal phase. **Chapter 5** is a short report on safety and efficacy aspects of early AA administration in preterm infants at two years of age. This is a follow up study in which we included infants earlier described in chapter 2. In this exploratory study, no negative effects of early AA administration on postnatal growth or handicap rates were found. However, despite a trend towards a better outcome in the group receiving AAs, no apparent clinical benefits were observed either in this exploratory study. Since neurodevelopmental outcome in preterm infants is dependent on multiple variables, of which only one is nutrition, larger studies are needed to define whether early aggressive AA administration will have a, perhaps subtle, effect on long-term, neurocognitive outcome.

Part II - early nutrition & antioxidant defense

Glutathione, a tripeptide and thus build up of AAs, is a very important antioxidant. Its requirements are likely to be increased as a result of exposure to so called 'reactive oxygen species', which are potential harmful intermediates in the consumption of oxygen. However, current feeding regimens not including substantial early AA administration are unlikely to meet these increased requirements. This results in oxidative stress and can cause serious morbidity.

In **Chapter 6**, we present a new method for the determination of glutathione (GSH) kinetics in erythrocytes of preterm infants. In this method, we used a liquid chromatography interface coupled to an isotope ratio mass spectrometer. This highly sensitive method requires only a very small sample volume. In addition, no derivatization is required, which is of benefit since GSH is very susceptible to artificial oxidation.

Chapter 7 describes an observational study in preterm infants in which we determined erythrocyte GSH concentrations and synthesis rates on postnatal day two (when receiving glucose only), and on postnatal day six, (when receiving both parenteral and enteral nutrition). In plasma, we measured advanced oxidized protein products (AOPP) and dityrosine as markers of oxidative stress. Towards day six both markers of oxidative stress increased significantly. However, this increase was not compensated for by an upregulation of GSH concentration and synthesis rate, despite an overall increase of plasma precursor AAs. This discrepancy results possibly from the two different compartments in which GSH (erythrocytes) and oxidative stress markers (plasma) were measured.

Early AA administration resulted in increased production of albumin as shown in Chapter 4. To investigate whether early AA administration stimulates GSH synthesis, we performed a clinical trial, described in **Chapter 8**, in which we determined GSH concentrations and synthesis rates in infants either receiving glucose only during the first two postnatal days, or infants receiving glucose and AAs directly following birth. In addition, we quantified levels of AOPP and dityrosine. The results showed that AA administration directly following birth increased GSH absolute synthesis rate by more than 70%. Our data are consistent, however, with higher GSH concentration rather than a higher fractional synthesis rate. Greater availability of GSH, nevertheless, did not bring down markers of oxidative stress.

As an explanation of the apparent relative decrease in GSH consumption, we speculated that the GSH fractional synthesis rate might have been increased already on the first day of life, and then have dropped as a result of negative feedback. Therefore, we performed another clinical trial, described in **Chapter 9**, in which we compared GSH kinetics in preterm infants in the direct postnatal phase (within four hrs postnatally) and on postnatal day two. We found that GSH concentration increased with postnatal age. However, contrary to our speculation, GSH fractional synthesis rate was not different between groups.

Cysteine is generally considered as the limiting substrate for GSH synthesis. However, in the past some raised concerns about its safety and bioavailability in preterm infants. In **Chapter 10** we studied the effects of doubling the amount of cysteine in a parenteral AA solution on GSH concentration and synthesis rate. Although it appeared metabolically safe, a high dose cysteine did not increase plasma cysteine concentrations. GSH concentration and synthesis rate were not altered upon the additional cysteine administration.

Alike preterm infants, sick term infants can be subjected to a high load of reactive oxygen species, for example during ventilation using additional oxygen. Therefore, their GSH requirements may be increased. In **Chapter 11**, we describe an observational study including infants suffering from perinatal asphyxia, infants requiring extra corporeal membrane oxygenation (ECMO), and relatively healthy, partially enterally fed preterm infants. Again, we determined GSH kinetics and we measured AOPP and dityrosine concentrations. Both AOPP and dityrosine concentrations were present in significant amounts in all groups. As compared to relatively healthy preterm infants, GSH concentrations and synthesis rates were not higher in asphyxiated infants, but GSH absolute synthesis rates were higher in infants on ECMO.

In **Chapter 12**, our findings are discussed in the context of relevant literature. In addition, recommendations for future research are made.

The main conclusions obtained from the studies described in this thesis are the following:

- Amino acid administration in preterm infants directly following birth is safe and converts a catabolic into an anabolic state by increasing protein synthesis. Energy for protein synthesis is not derived from increased glucose oxidation.
- Early amino acid administration in preterm infants appears to be safe with regard to neurodevelopmental outcome at two years of age.
- Decreased glutathione concentration in erythrocytes from preterm infants is due to nutritional deprivation and can be stimulated by early amino acid administration. In addition, in response to early amino acid administration, glutathione consumption seems to be decreased. However, levels of plasma markers of oxidative stress are not diminished.
- High dose cysteine appears safe but does not increase glutathione concentration or synthesis rate in erythrocytes of preterm infants as compared to a lower dose.

Samenvatting

Voeding wordt in toenemende mate erkend als een belangrijke pijler in de behandeling van prematuur geboren kinderen. Dit proefschrift bevat een aantal studies waarin het effect van het toedienen van aminozuren direct na geboorte op de aanmaak van eiwitten en antioxidanten wordt bestudeerd. Aminozuren zijn de bouwstenen waaruit eiwitten en bepaalde antioxidanten zijn opgebouwd.

In **Hoofdstuk 1** wordt een algemene introductie gegeven over voeding voor premature pasgeborenen in de eerste levensfase. Premature pasgeborenen zijn voor het grootste deel van hun voedingsintake afhankelijk van parenterale voeding in de eerste levensperiode. Parenterale voeding is voeding die via een infuus wordt toegediend.

Helaas ging het gebruik van de eerste generatie aminozuuroplossingen gepaard met een aantal serieuze bijwerkingen op het metabolisme (stofwisseling). Hoewel de huidige oplossingen deze bezwaren in principe niet meer kennen, zijn veel kinderartsen nog terughoudend om aminozuren (in een significante hoeveelheid) toe te dienen in de eerste levensdagen. Het gevolg is dat deze premature pasgeborenen in deze periode meer eiwitten afbreken dan dat ze aan kunnen maken. Het is niet moeilijk voor te stellen dat dit op korte termijn negatieve gevolgen heeft en wellicht zelfs ook op langere termijn. In dit proefschrift worden studies gepresenteerd waarin we de veiligheid en de effectiviteit bestuderen van het direct na geboorte toedienen van aminozuren via het infuus. Hierbij kijken we naar hoe de aminozuren verdragen worden. Daarnaast kijken we vooral naar effecten op aanmaak van eiwitten en antioxidanten.

Deel I – vroege voeding & eiwitmetabolisme

In **Hoofdstuk 2** wordt een onderzoek beschreven waarin algemene aspecten van veiligheid en effectiviteit worden bestudeerd van parenterale aminozuurtoediening direct na de geboorte in een grote groep van premature pasgeborenen met een geboortegewicht van onder de 1500 gram. Een controlegroep kreeg alleen koolhydraten (glucose) toegediend via het infuus gedurende de eerste 2 levensdagen. Qua veiligheid keken we onder andere naar de pH en de base excess, een maat voor de zuurgraad van het bloed. Afgezien van een wat verhoogd ureum gehalte van het bloed vonden we geen significante verschillen tussen beide groepen. Ureum wordt gevormd bij de verbranding van aminozuren waarbij energie vrijkomt. Het hogere ureumgehalte dient naar ons idee echter niet geïnterpreteerd te worden als intolerantie ten opzichte van aminozuren, maar als een teken dat de aminozuren verbrand worden om in de energiebehoefte te voorzien. Verder vonden we in deze studie dat door het toedienen van aminozuren een anabole voedingstoestand wordt verkregen, wat wil zeggen dat er meer eiwit wordt

aangemaakt dan dat er wordt afgebroken. Ook waren de concentraties van enkele essentiële aminozuren hoger. Essentiële aminozuren zijn aminozuren die (nog) niet door het lichaam zelf gemaakt kunnen worden en dus via voeding dienen te worden ingenomen. Concluderend lijkt het toedienen van aminozuren direct na geboorte veilig en resulteert het in een anabole voedingstoestand.

In **Hoofdstuk 3** bestuderen we hoe het anabolisme na het toedienen van aminozuren in de eerste levensdagen verkregen wordt. Met behulp van met stabiele isotopen gelabelde aminozuren werd aangetoond dat dit door toegenomen eiwitaanmaak komt, terwijl de eiwitafbraak niet geremd wordt door aminozuurtoediening. In tegenstelling tot premature pasgeborenen wordt anabolisme bij oudere kinderen en volwassenen voornamelijk bewerkstelligd door het remmen van de eiwitafbraak en juist in mindere mate door het stimuleren van eiwitaanmaak. Dit verschil heeft mogelijk te maken met het vinden van een balans tussen afbraak en aanmaak in het metabool zeer actieve premature pasgeboren kind. In deze studie werd ook gekeken naar verbranding van glucose. Immers, eiwitaanmaak is arbeidsintensief. Er werd echter geen verschil gevonden in glucose verbranding, wat suggereert dat de energie nodig voor eiwitaanmaak niet wordt gewonnen uit een toegenomen glucose verbruik.

Terwijl in hoofdstuk 3 niet naar een specifiek eiwit werd gekeken, werd in **Hoofdstuk 4** de aanmaak van albumine bestudeerd, het in bloedplasma meest voorkomende eiwit. Er werd gevonden dat zowel de hoeveelheid albumine als de aanmaaksnelheid ervan significant toenam na het toedienen van aminozuren. Aangezien albumine een eiwit is met zeer belangrijke functies, is deze bevinding klinisch belangrijk.

In de voorgaande hoofdstukken bestudeerden we effecten van aminozuurtoediening in de eerste levensfase. In **Hoofdstuk 5** wordt een exploratieve studie beschreven die naar lange termijn veiligheid en effectiviteit kijkt van aminozuurtoediening naast glucose versus alleen glucose gedurende de eerste twee levensdagen. Kinderen werden op de leeftijd van 2 jaar gezien. In deze studie werden dezelfde kinderen geïncludeerd als beschreven in hoofdstuk 2. Op de leeftijd van 2 jaar werden geen negatieve effecten gevonden van het vroeg toedienen van aminozuren op het voorkomen van lichamelijke of verstandelijke handicaps. Ook werd er echter geen (significant) betere uitkomst gevonden tussen kinderen die glucose en aminozuren versus alleen glucose ontvangen hadden. Aangezien neurologische ontwikkeling afhankelijk is van meerdere variabelen, waarvan voeding er een is, zijn grotere studies nodig om aan te tonen of vroege toediening van aminozuren klinisch significante positieve effecten heeft op neurologische ontwikkeling.

Deel II – vroege voeding & antioxidanten

Glutathion, opgebouwd uit aminozuren, is een zeer belangrijke antioxidant. Antioxidanten maken afvalstoffen onschadelijk die vrijkomen bij stofwisselingsprocessen, zoals vrije radicalen bij het verbruik van zuurstof door het lichaam. De behoefte aan antioxidanten en dus glutathion in premature pasgeborenen is verhoogd als gevolg van zuurstoftoediening, ziekten, infecties, etc. Echter, zonder aminozuurtoediening zijn er mogelijk te weinig bouwstenen om in de behoefte aan glutathion te voorzien. Er onstaat dan een situatie die we oxidatieve stress noemen en die tot ernstige ziektebeelden kan leiden.

In **Hoofdstuk 6** presenteren we een nieuwe methode om glutathion aanmaak te bepalen in rode bloedcellen van premature pasgeborenen. Deze op massa spectrometrie gebaseerde methode is zeer nauwkeurig en behoeft slechts een minimale hoeveelheid bloed.

In **Hoofdstuk 7** wordt een observationele studie beschreven in premature pasgeborenen met een geboortegewicht kleiner dan 1000 gram waarin glutathion concentraties en aanmaaksnelheid worden bepaald op levensdag twee (wanneer kinderen slechts glucose kregen) en op dag zes (wanneer kinderen zowel parenterale voeding als voeding via de natuurlijk weg werd toegediend). In het bloedplasma werd de mate van oxidatieve stress bepaald. Op zowel dag twee als dag zes waren de oxidatieve stress markers verhoogd. Dit werd echter niet gecompenseerd door een toegenomen glutathion productie. Mogelijk wordt deze discrepantie veroorzaakt door het feit dat glutathion bepaald werd in rode bloedcellen, terwijl oxidatieve stress bepaald werd in bloedplasma door gebrek aan voldoende rode bloedcellen.

Direct na geboorte toedienen van aminozuren resulteerde in een toegenomen productie van albumine, zoals aangetoond in hoofdstuk 4. Om te onderzoeken of ook glutathion aanmaak wordt gestimuleerd, werd een studie uitgevoerd, beschreven in **Hoofdstuk 8**, waarin glutathion concentraties en aanmaaksnelheid werden bepaald in premature pasgeborenen die of naast glucose ook aminozuren toegediend kregen of die alleen glucose kregen in de eerste twee levensdagen. Ook bepaalden we weer oxidatieve stress markers. De resultaten lieten zien dat de absolute glutathion productie met 70% toenam. Dit werd echter niet bewerkstelligd door een hogere fractionele aanmaaksnelheid, als wel door een hogere concentratie van glutathion. Dit suggereert dat het toedienen van aminozuren resulteert in een relatieve afname van het glutathion verbruik. De grotere glutathion beschikbaarheid leidde verder niet tot een vermindering van oxidatieve stress markers in bloedplasma.

Als verklaring voor de relatieve afname van het verbruik van glutathion, werd gespeculeerd dat de fractionele aanmaaksnelheid al verhoogd was op de eerste levensdag, waarna het afnam als gevolg van voldoende beschikbaarheid. Om dit te onderzoeken, onderzochten we glutathion concentraties en aanmaaksnelheid al binnen enkele uren na de geboorte en vergeleken dit met waardes verkregen op levensdag twee. Dit is beschreven in **Hoofdstuk 9**. Terwijl de concentratie naar levensdag twee duidelijk toenam, vonden we geen toegenomen fractionele aanmaaksnelheid direct na geboorte, wat suggestief is voor een afname van glutathion verbruik.

Van cysteine, een van de drie aminozuren waaruit glutathion is opgebouwd, wordt algemeen aangenomen dat het de limiterende bouwsteen is voor de glutathion aanmaak. Het is van de drie aminozuren in de laagste concentratie in bloed aanwezig. De veiligheid van het toedienen van het losse aminozuur cysteine heeft volgens sommigen mogelijk bijwerkingen tot gevolg. Daarnaast is het onzeker of het lichaam het in losse vorm kan verwerken. In **Hoofdstuk 10** wordt een studie beschreven waarin we een groep premature pasgeborenen de standaard hoeveelheid cysteine aanwezig in de aminozuuroplossing toedienden en vergelijken dit met een groep die de dubbele hoeveelheid ontving. Hoewel er geen aanwijzingen werden gevonden voor intolerantie van de hogere concentratie cysteine, was de concentratie van cysteine in bloedplasma niet hoger in de groep die meer cysteine ontving. Ook waren glutathion concentratie en aanmaaksnelheid niet verhoogd.

Net als premature pasgeborenen, kunnen zieke op tijd geboren pasgeborenen worden blootgesteld aan grote hoeveelheden vrije radicalen, zoals bij blootstelling aan hoge concentraties zuurstof als onderdeel van hun behandeling. Hierdoor is hun behoefte aan antioxidanten als glutathion mogelijk vergroot. In **Hoofdstuk 11** wordt een observationele studie beschreven waarin pasgeborenen met een periode van zuurstofgebrek rondom de geboorte, pasgeborenen aan de hart-long machine en relatief gezonde premature pasgeborenen werden geïncludeerd. Bij deze kinderen werden weer glutathion concentraties en aanmaaksnelheid als ook oxidatieve stress markers in bloedplasma bepaald. In alle groepen leek er sprake van oxidatieve stress. Vergeleken met premature pasgeborenen was er geen verschil in glutathion concentratie en aanmaaksnelheid in pasgeborenen met zuurstofgebrek rondom de geboorte. Zowel concentratie als aanmaaksnelheid van glutathion was echter beduidend groter in pasgeborenen aan de hart-long machine.

In **Hoofdstuk 12** worden de bevindingen in dit proefschrift bediscussieerd in de context van relevante literatuur. Daarnaast worden suggesties gedaan voor toekomstig onderzoek.

De belangrijkste conclusies volgend uit dit proefschrift zijn als volgt:

- Aminozuurtoediening aan premature pasgeborenen direct na de geboorte is veilig en resulteert in een anabole voedingstoestand door middel van het stimuleren van de eiwitproductie. De energie nodig voor toegenomen eiwitproductie wordt niet verkregen uit verbranding van glucose.
- Aminozuurtoediening direct na geboorte in premature pasgeborenen lijkt veilig voor wat betreft de neurologische ontwikkeling op tweejarige leeftijd.
- Een lage glutathion concentratie in rode bloedcellen van premature pasgeborenen in de eerste levensfase is het gevolg van een tekort aan bouwstenen en kan worden gestimuleerd door het toedienen van aminozuren. Tevens lijkt het verbruik van glutathion door toediening van aminozuren af te nemen. Desondanks zijn oxidatieve stress markers in bloedplasma onverminderd aanwezig.
- Het toedienen van een hogere dosis cysteine lijkt veilig maar leidt niet tot een verhoogde aanmaaksnelheid van glutathion in rode bloedcellen van premature pasgeborenen in vergelijking tot een lagere dosis.

Dankwoord

Een proefschrift schrijven is een aparte ervaring. Het is hollen, soms slenteren, af en toe zelfs stilstaan. Het is *multi tasken* ten voeten uit! In dat kader is het overigens niet verwonderlijk dat het 'hora est' voor vrouwelijke promovendi ruim een jaar eerder klinkt.
Zoals uit onderstaande zal blijken is een proefschrift de uitkomst van een gezamenlijke inspanning. Graag wil ik daarom op deze plaats de volgende personen bedanken.

Allereerst dank ika de kinderen en hun ouders die belangeloos meegewerkt hebben aan de verschillende onderzoeken. Zonder onderzoek geen vooruitgang. De resultaten beschreven in dit proefstuk zullen zeker direct of indirect een bijdrage leveren aan het verbeteren van de zorg voor prematuur geboren baby's.

Prof. dr. J.B. van Goudoever, beste Hans. Ondanks je volle agenda zijn laagdrempeligheid, enthousiasme en scherpzinnigheid sleutelwoorden in je omgang met promovendi. Ik heb het als zeer prettig ervaren dat je me in het onderzoek vrij liet, maar altijd weer bijstuurde wanneer nodig. De vele congresbezoeken waren met recht fantastische kroenten in de pap, met afgelopen voorjaar met de hele neo naar Hawaii als hoogtepunt!

De leden van de Kleine Commissie, prof. dr. D. Tibboel, prof. dr. A.J. van der Heijden en prof. dr. H.J.G. Boehm, dank ik hartelijk voor het beoordelen van het manuscript. Prof. Tibboel, zeer veel dank voor het bieden van de mogelijkheid om onderzoek te doen op de toenmalige intensive care chirurgie.

Prof. dr. G. Buonocore, Prof. dr. E.A.P. Steegers, prof. dr. H.N. Lafeber en prof. dr. H.P. Sauerwein bedank ik voor het zitting nemen in de Grote Commissie.

Professor Buonocore and Mariangela Longini. Thank you very much for introducing our study group into the world of oxidative stress markers. I am confident that our ongoing collaboration will lead to exciting data.. and hopefully some more visits to Siena of course!

Alle artsen en verpleegkundigen van de afdeling neonatologie wil ik bedanken voor hun hulp bij het uitvoeren van de verschillende studies. Er wordt veel onderzoek gedaan op de afdeling neonatologie, wat regelmatig vraagt om extra inspanningen van het personeel. Van gaasjes in de luier om urine op te vangen en het afnemen van bloedmonsters tot hulp bij het includeren van patiënten. Zeer bedankt!

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De apotheek. Er zijn heel wat stabiele isotopen opgelost en cysteine testoplossingen gemaakt. En altijd in een rap tempo! Hiervoor ben ik Andras Vermes zeer erkentelijk. Ook de verschillende trialapothekers wil ik bedanken voor hun inzet voor het importeren van de cysteine oplossingen, wat geen gemakkelijk klus bleek. Met name wil ik Satu Siiskonen op deze plaats daarvoor bedanken.

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Chris, vriend en collega, a.k.a. Jut. Samen zijn we níet naar Madrid gegaan, maar wel 4 jaar langer op de Sk-2210 gebleven. We zijn aardig wat beren op de onderzoeksweg tegengekomen, maar de voetbalsessies op de kamer, Coenense biertjes en de vele tripjes rondom congresbezoeken hebben zeker voor de nodige ontspanning gezorgd.

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Lieve Susan. Vooral de laatste maanden was de studeerkamer voor mij the place to be. Op jou kon ik gelukkig elke keer weer rekenen als ik het boekje even moe was. Samen met jou gaat de blik vanaf nu weer op de toekomst!

List of Publications

CHP van den Akker, **FWJ te Braake**, JB van Goudoever.
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Amino acid administration to premature infants directly after birth.
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CHP van den Akker, **FWJ te Braake**, JLD Wattimena, G Voortman, H Schierbeek, A Vermes, JB van Goudoever.
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Glutathione synthesis rates and oxidative stress in extremely low birth weight infants in the first week of life.
Submitted.

FWJ te Braake, H Schierbeek, A Vermes, JGM Huijmans, JB van Goudoever.
High dose cysteine does not stimulate glutathione synthesis in parenterally fed preterm infants.
Submitted.

D Rook, **FWJ te Braake**, H Schierbeek, M Longini, G Buonocore, JB van Goudoever.
Glutathione synthesis rates in the immediate postnatal phase
Submitted.

FWJ te Braake, CHP van den Akker, N Weisglas-Kuperus, JB van Goudoever.
Long term safety and efficacy aspects of early amino acid administration in preterm infants.
Submitted.

Curriculum Vitae

Frans te Braake was born on December 19th, 1978 in Groningen, the Netherlands. After completing grammar school at Maartenscollege in Haren, he started medical school at Erasmus University Rotterdam in 1997.

In 2001, he interrupted his studies for one year to become a full time member of the board of student fraternity S.S.R.-Rotterdam. In the same year, he was board member of the Society for Medical Interfacultary Conventions (MIC).

From 2003 until 2004, he participated in a research project on nutritional intervention for preterm infants as a graduation project at the division of neonatology of the department of pediatrics in Erasmus MC - Sophia Children's Hospital in Rotterdam. In 2004 he postponed his rotations for his medical training to start working as a PhD-student (supervisor Prof. dr. J.B. van Goudoever), which lead to the present dissertation.

In 2008 he resumed his medical training, which he will finish in 2009.

Frans lives together with Susan de Grijp in Rotterdam.

Portfolio

Courses:

- 2007: SPR-RC (Society for Pediatric Research – Research Conference) on health promoting effects of early nutrition, The Woodlands, TX, USA.
- 2006: Intensive Course in Tracer Methodology in Metabolism, Stockholm, Sweden.
- 2005: Nihes Statistics Course: Classical Methods for Data Analysis, Rotterdam, the Netherlands.

Awards:

- 2007: Travel award for attending the SPR-Research Conference in The Woodlands, TX, USA.
- 2005: NVVL (Network for Food Experts) Award for best scientific thesis.
- 2004: 'Jan C. Molenaar' award for best presentation (selected by the Scientific Advisory Council of the Sophia Foundation for Scientific Research).
- 2003: Best scientific poster presentation, NVK (Dutch Society of Pediatrics) Convention.

Memberships:

- 2007: BASIS (BeNeLux Association for Stable Isotope Scientists).
- 2007: ASN (American Society for Nutrition), research interest section: Energy and Macronutrient Metabolism.

Conferences:

- 2008: -High dose cysteine does not improve antioxidant defense mechanism in preterm infants. Soc.Ped.Res.-Ped.Assoc.Soc (SPR-PAS), Honolulu, Hawaii, USA. (Poster)
  -Glutathione synthesis rates and oxidative stress markers in infants supported by extracorporeal membrane oxygenation. SPR-PAS, Honolulu, Hawaii, USA. (Poster)
- 2007: -Effects of amino acid administration on glutathione kinetics and oxidative stress markers in preterm infants. SPR-Research Conference, The Woodlands, TX, USA. (Poster)
  -Effects of early amino acid adminstration on glutathione kinetics and oxidative stress in preterm infants. Eur.SPR, Prague, Czech Republic. (Oral)

-Glutathione kinetics and oxidative stress in preterm neonates in the first week of life. Eur.SPR, Prague, Czech Republic. (Poster)

-Glutathione kinetics and oxidative stress markers during early neonatal life. Exper.Biol., Washington DC, USA. (Oral)

-Glutathione synthesis rates in premature neonates. BASIS, Leuven, Belgium. (Oral)

- 2006: -Glutathione kinetics in premature infants. European Academy of Paediatrics, Barcelona, Spain. (Poster)

-Less stress. Sophia Research Day, Rotterdam, the Netherlands. (Oral)

- 2005: -Leucine and glucose metabolism after amino acid administration directly postnatally in premature neonates with birth weights < 1500 grams. SPR-PAS, Washington DC, USA. (Poster)

- 2004: -Amino acid administration directly from birth onwards in VLBW infants is safe and results in anabolism. SPR-PAS, San Francisco, CA, USA. (Poster)

-Amino acid administration directly postpartum in premature infants. Neonatology Retreat Days, Leiden, the Netherlands. (Oral)

-Leucine metabolism after amino acid administration directly postnatally to premature infants <1500 grams. NVK (Dutch Pediatr. Soc.) convention, Veldhoven, the Netherlands. (Poster)

- 2003: -Amino Acid Administration directly postpartum to prematurely born infants. NVK convention, Veldhoven, the Netherlands. (Poster)