Perinatal asphyxia and oxidative stress

Studies in preterm IUGR pregnancies and term acute asphyxia

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Perinatale asfyxie en oxidatieve stress

Studies in premature IUGR zwangerschappen en a terme acute asfyxie
(met een samenvatting in het Nederlands)

Proefschrift

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To Mum and Dad

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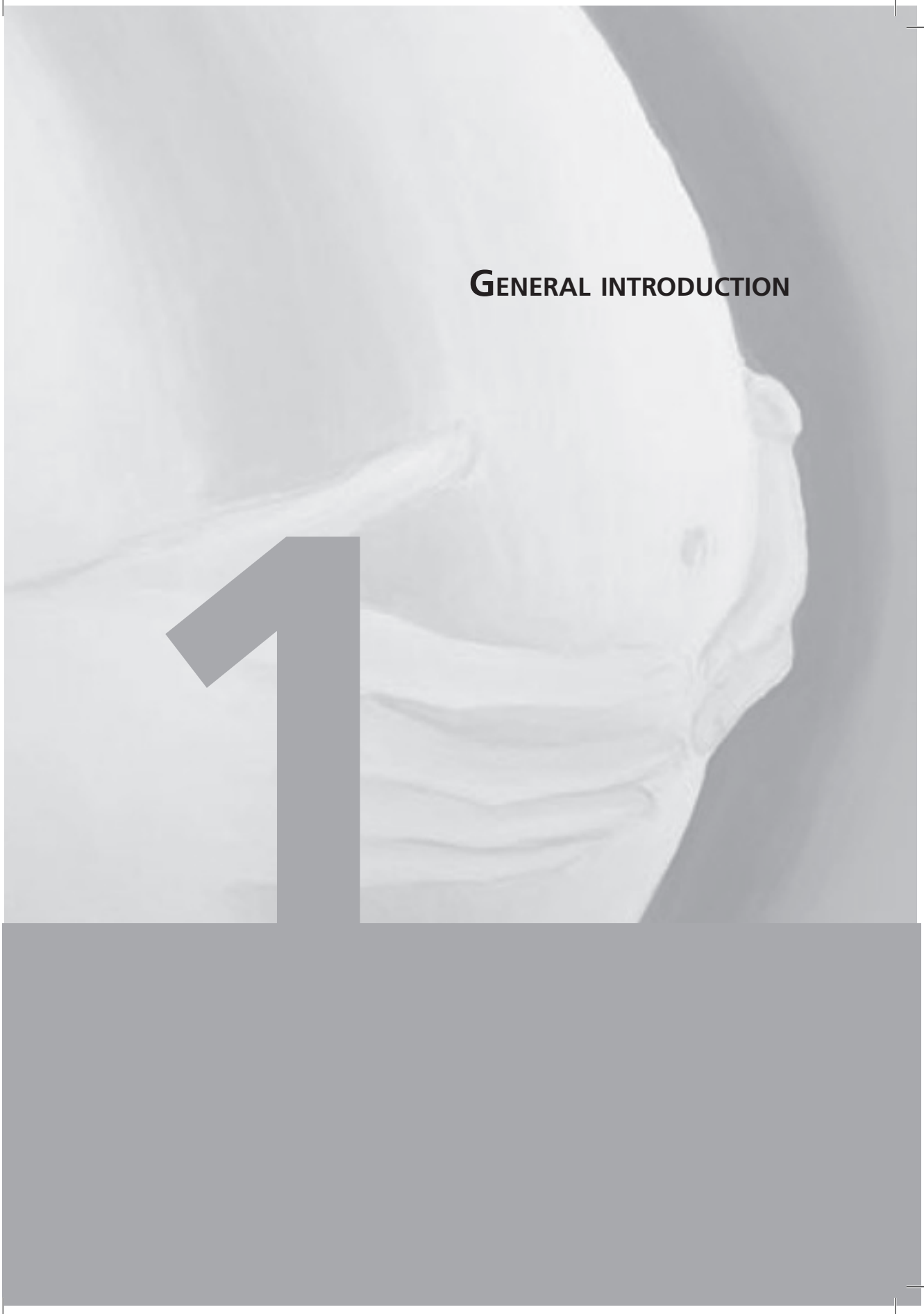

1 General introduction

Preterm intrauterine growth restriction (IUGR) and term asphyxia remain major causes of perinatal mortality and morbidity (1-4) and lifelong neurodevelopmental morbidity, including cerebral palsy, learning disabilities and mental retardation (4-7). For preterm IUGR foetuses, the incidence of neonatal death has been reported to be 12-23% (8) with an odds ratio as high as 6 to 9 times that of appropriately grown foetuses of the same gestational age (9). Abnormal neurodevelopment at 2 years of age has been reported to occur in 6-18% of preterm IUGR foetuses (10). For term asphyxia, approximately 15% to 20% of asphyxiated infants who show signs of hypoxic-ischemic encephalopathy die during the newborn period, and of the survivors, 25% has permanent neuropsychological deficits (4).

While the complex pathophysiology of these severe conditions differs, oxidative stress has been implicated in both preterm IUGR and term asphyxia (11-17). Oxidative stress results from a failure in the balance between pro- and antioxidant mechanisms (18). Small amounts of reactive oxygen species (ROS) are very commonly formed in the body and play an important role in normal physiology. Greatly increased ROS production, however, due to for instance hypoxia and ischemia/reperfusion (19;20), can be harmful. ROS are reactive compounds with an uneven number of electrons in their orbit which makes them unstable. To generate stability, ROS donate or take electrons from other molecules to pair their electrons. Pairing of electrons results in irreversible damage to cellular components such as fatty acids of membrane lipids or DNA, ultimately resulting in cell death. Indirectly, ROS cause additional damage by promoting further ROS production and initiating ROS chain reactions (18). ROS production is therefore regulated by antioxidant mechanisms which include endogenous enzymes that catalyse the removal of ROS and non-enzymatic anti-oxidant compounds that directly scavenge free radicals. The balance between these pro- and antioxidant mechanisms is vital for the maintenance of health.

Part 1
Oxidative stress and other factors affecting outcome of the preterm IUGR foetus

Abnormal placentation has been linked with oxidative stress (21). Abnormal placentation in early pregnancy can result in placental insufficiency (and subsequent IUGR) and/or maternal hypertensive disease of pregnancy (22;23).
Placental insufficiency has been hypothesised to cause chronic intrauterine stress which could lead to accelerated foetal lung maturation by stimulation of foetal glucocorticoid production (24). This may result in a reduced incidence of respiratory distress syndrome (RDS) in preterm IUGR infants. Several groups have been unable to confirm this hypothesis (25-27) and, contrary to expectation, two of these groups found a higher RDS incidence in IUGR infants (25;26). Hence, chronic intrauterine stress due to placental insufficiency does not seem to reduce RDS incidence. RDS is a syndrome of multifactorial aetiology with the most important factor being surfactant deficiency. Oxidative stress plays a role in RDS pathophysiology by causing lung tissue damage of the alveolar type II cell, inhibiting surfactant metabolism and causing surfactant inactivation (28). It is conceivable that chronic intrauterine *oxidative* stress due to placental insufficiency may cause lung damage. Unfortunately, the studies mentioned above (25-27) did not take the maternal condition into account. This is important because preterm IUGR and hypertensive disease of pregnancy occur concomitantly in many cases and neonates from pregnancies complicated by HELLP syndrome have been shown to have an increased RDS incidence as compared to neonates born to mothers without hypertension or to neonates born to mothers with pregnancy induced hypertension (29;30). Furthermore, numbers were small (27), no correction for potential confounders was performed (26) and wide gestational age ranges were studied resulting in heterogeneous study populations (25-27).

In the foetal lung, glucocorticoids enhance surfactant synthesis, increase lung compliance, promote structural and functional maturation, and stimulate reabsorption of lung fluid. Endogenous glucocorticoid production rises before transition from intrauterine to extrauterine life. Prematurely born infants may miss the pre-partum exposure to this rise in glucocorticoid which makes them susceptible to developing RDS. Antenatal steroid treatment for reduction of RDS has been studied extensively and general consensus is that this treatment should be administered to women at risk of preterm birth (31-33). IUGR foetuses, however, were excluded from the large trials due to early reports by Liggins et al that steroids might be harmful in pregnancies complicated by IUGR

and hypertension (increase of foetal death rate) (34). In IUGR animal models, antenatal steroids have been shown to have harmful effects, which include a reduction of cerebral growth, alteration of foetal (cerebral) blood flow and brain damage (35-37). Although the clinical studies mentioned above, keeping the limitations in mind, have not been able to confirm the hypothesis that RDS is decreased in IUGR (25-27), there is concern about beneficial effects of antenatal steroid administration in this specific subgroup and it is uncertain if IUGR foetuses should be treated with antenatal steroids.

For clinicians, it is vital to know which parameters should be taken into account when deciding on whether or not to administer antenatal steroid treatment and when deciding on the optimal time of delivery of the IUGR foetus. The benefits of prolonged intrauterine maturation have to be weighed against complications that may occur due to prolonged malnutrition and hypoxia. Antenatal predictors for adverse neonatal outcome after IUGR have therefore been studied extensively (10;26;38-48). In these studies, GA, birth weight and antenatal Doppler indices have been shown to be important predictors for intact neonatal survival. Unfortunately, the placenta, a key organ dictating the intrauterine environment, has never been studied. Even more importantly, predictors for long term neurodevelopmental outcome were never assessed. In our opinion, information on expected neurodevelopmental outcome of the individual IUGR foetus is of vital significance when counselling prospective parents and deciding on the optimal time of delivery. Many monitoring techniques for determining the optimal time of delivery are used in the management of IUGR which include longitudinal assessment of foetal biometry, amniotic fluid volume, Doppler indices, foetal heart rate pattern, foetal movements and foetal breathing (reviewed in (49-51)). Several groups have investigated whether magnetic resonance spectroscopy (MRS) could also be valuable in estimating foetal wellbeing (52-55). Amniotic fluid lactate and creatinine can be measured by MRS and amniotic fluid lactate concentration has been suggested to reflect the extent of foetal lacticemia. In IUGR pregnancies, the amount of amniotic fluid is often reduced and we therefore speculated that the foetal lactate concentration should be corrected for the amount of amniotic fluid by calculation of the lactate to creatinine (L:C) ratio. Huang and colleagues have shown that measurement of the urinary L:C ratio helps identify asphyxiated newborns at high risk of developing hypoxic-ischemic encephalopathy (56). The amniotic fluid L:C ratio may provide us with a more reliable estimation of the extent of foetal lacticemia and may thereby aid in the determination of the optimal time of delivery of the IUGR foetus.

In summary, it remains unclear if placental insufficiency and/or HELLP syndrome *independently* influence clinical respiratory outcome (as indicated by RDS incidence) or foetal lung maturity (as indicated by the amniotic fluid lecithin to sphingomyelin (L/S) ratio) of preterm small for gestational age (SGA) foetuses. If RDS incidence is in fact increased in preterm neonates born to mothers with HELLP syndrome, one may expect to find higher levels of oxidative stress in these neonates. It would be interesting to know if placental histological and oxidative stress parameters differ between the various disease states, considering that abnormal placentation is a key factor in the development of both IUGR and maternal hypertensive disease of pregnancy. To our knowledge, very little research has been performed in this area and specifically in *preterm* placentae from pregnancies complicated by HELLP syndrome. Finally, it remains unclear whether IUGR foetuses should receive antenatal steroids for foetal lung maturation and which predictors are of importance for long term neurodevelopmental outcome of these foetuses.
Therefore, the aim of the first part of this thesis was to study oxidative stress and other factors affecting outcome of the IUGR foetus.

Aims of part 1 of this thesis

- To determine the RDS incidence in preterm SGA neonates with or without abnormal foetal umbilical artery Doppler and/or maternal hypertension (Chapter 2)
- To study the amniotic fluid L/S ratio in preterm SGA foetuses from pregnancies complicated by placental insufficiency or maternal hypertension (Chapter 3)
- To study oxidative stress and inflammation in preterm neonates of preeclamptic mothers with HELLP syndrome (Chapter 4)
- To correlate placental histo(immuno)pathology to the extent of maternal hypertensive disease and foetal Doppler ultrasound findings (Chapter 5)
- To review the available literature on antenatal steroid treatment of IUGR foetuses (Chapter 6)
- To determine whether amniotic fluid lactate concentration or the amniotic fluid L:C ratio is a better predictor of foetal lacticemia (Chapter 7)
- To study the outcome of IUGR infants at 2 years of age in relation to antenatal, perinatal and neonatal factors (Chapter 8)

Part 2
Therapeutic approach for antenatal reduction of foetal oxidative stress

Ultimately, it would be of great clinical significance if an intrauterine therapeutic approach could be designed to reduce oxidative stress in both the term asphyxiated infant and preterm IUGR foetus. Studies on intrauterine treatment to reduce oxidative stress due to abnormal placentation have focused on the non-enzymatic antioxidant vitamins C and E that are commercially available and are easily administered to humans. Unfortunately, both compounds have been proven to be ineffective in human studies (57-59).

Fortunately, more extensive research on reduction of oxidative stress following term asphyxia has been performed. Moderate hypothermia has been shown to provide neuroprotection in moderately asphyxiated term newborns (60;61). However, because injury following hypoxia-ischemia and reperfusion is complex, combined therapy targeting various harmful pathways may be of additional value (by for instance combining hypothermia with a pharmacological means of neuroprotection). Many promising pharmacological compounds (including selective iNOS and nNOS inhibition (62-66), early NFkappaB inhibition (67;68), erythropoietin (69-71), melatonin (72), allopurinol (17;73) and xenon (74;75)) have been studied in neonatal animal models of hypoxic-ischemic brain damage. Encouraging results of translational pharmacological intervention in *human* neonates were reported by van Bel et al. A pilot study by this group showed that postnatal allopurinol treatment of asphyxiated babies reduced free radical formation and maintained cerebral blood flow and electrical brain activity (76). Following these positive results, a multicentre randomised trial was designed in which severely asphyxiated newborns were treated with allopurinol or placebo. After inclusion of 32 babies, mortality was substantial (>73%) and it was feared that allopurinol therapy was not effective in this group of severely asphyxiated infants which was confirmed by an interim analysis (77). The reason for this lack of effect may be the late point in time of allopurinol administration (median time 3 to 4 hours after birth). In pregnancy complicated by foetal hypoxia, treating the foetus via the mother, rather than the neonate, with allopurinol might be more effective. Maternal allopurinol treatment during *uncomplicated* term labour has previously been shown to be feasible (78). Thus far, maternal allopurinol treatment during foetal hypoxia has not been studied in animals or humans.

Allopurinol might also be effective in reducing oxidative stress in pregnancies complicated by IUGR. However, before chronic intrauterine allopurinol administration for IUGR can be considered, the effects of maternal allopurinol treatment should be studied in a

term animal model of foetal asphyxia. Furthermore, if treatment seems to be effective in this model, the effects of a single dose of maternal allopurinol in humans should be evaluated during acute foetal distress in complicated term labour. Only if maternal allopurinol treatment is proven to be safe and effective in humans, should chronic intrauterine treatment for IUGR be considered. Naturally, once more this should initially be studied in an IUGR animal model.

Thus, the aim of the second part of this thesis was to study the effect of maternal allopurinol treatment in a near term foetal sheep model of foetal asphyxia and in a human trial during foetal distress. These studies are a first step toward developing an intrauterine approach for reduction of foetal oxidative stress.

Aims of part 2 of this thesis

- To review the literature on possible therapeutic approaches in term asphyxia (Chapter 9)
- To study the effect of maternal allopurinol treatment on umbilical blood flow in a near term foetal sheep model of foetal asphyxia (Chapter 10)
- To study the effect of maternal allopurinol treatment on foetal cardiac oxidative stress in a near term foetal sheep model of foetal asphyxia (Chapter 11)
- To study placental passage of maternal allopurinol treatment during term human labour complicated by foetal distress and to study its effect on umbilical cord S-100B levels (a brain injury marker protein) and oxidative stress parameters (Chapter 12)

PART 1

RESPIRATORY OUTCOME IN PRETERM SMALL FOR GESTATIONAL AGE FOETUSES WITH OR WITHOUT ABNORMAL UMBILICAL ARTERY DOPPLER AND/OR MATERNAL HYPERTENSION

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Abstract

Objective

To study respiratory outcome of preterm small for gestational age (SGA) foetuses with or without signs of intrauterine growth restriction due to placental insufficiency and with or without maternal hypertension.

Methods

Retrospective study in 187 neonates with birth weight <10th percentile and gestational age <34 weeks. Results from umbilical artery Doppler velocimetry were used to identify the abnormal Doppler subgroup.

Results

No significant difference in respiratory outcome between SGA foetuses with normal or abnormal (SGA-A) umbilical artery Doppler examination existed. Within the SGA-A group, the respiratory distress syndrome (RDS)-incidence (OR=5.6, 95%CI=1.7-18.3), RDS-grade (OR=6.7, 95%CI=1.2-38.5) and need for surfactant (OR=5.3, 95%CI=1.1-24.4) were higher in infants of women with haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome as compared to those of normotensive mothers.

Conclusions

Lung maturation is not accelerated with placental insufficiency. SGA-A foetuses of mothers with HELLP syndrome have a significantly poorer respiratory outcome than those with healthy mothers. Possibly, foetuses of mothers with HELLP syndrome are subjected to 'oxidative stress' causing lung damage rather than lung maturation.

Introduction

Uteroplacental dysfunction accounts for the majority of cases of intra-uterine growth restriction (IUGR) (79). Foetuses with IUGR represent a subset of those designated as small for gestational age (SGA) (27). SGA foetuses also include those that are small due to constitutional or genetic causes. Foetuses with IUGR are difficult to differentiate from other SGA foetuses (27). With growth restriction due to placental insufficiency, there is elevated impedance to blood flow in the placenta (80). This is reflected by abnormal umbilical artery velocimetry with or without increased blood flow to the foetal brain ('brain-sparing') (27;81). Therefore, foetuses with abnormal Doppler waveform patterns are likely to represent the true IUGR infant.

It has been postulated that IUGR foetuses have accelerated pulmonary maturation, because they are subjected to prolonged intrauterine stress through diminished oxygen delivery due to placental dysfunction (24). Various studies have tested this hypothesis in SGA versus appropriate for gestational age (AGA) foetuses. The results from these studies are non-conclusive with the risk of respiratory distress syndrome (RDS) being lower (82), equal (9;79) or higher (83-87) in SGA infants.

Thus far, to our knowledge, no comparison has been made of respiratory outcome between preterm SGA foetuses with abnormal Doppler findings (SGA-A; likely to represent the true IUGR foetus) and premature SGA foetuses with normal Doppler (SGA-N). Earlier studies in this field covered a wide gestational age range (25-42 weeks) and did not take possible effects of maternal (hypertensive) disease on respiratory outcome of the preterm newborn infant into account (25-27). We hypothesise that:

1) the premature SGA-A foetus has less respiratory morbidity compared to the SGA-N foetus of the same gestational age (GA) due to accelerated pulmonary maturation during placental insufficiency,

2) the SGA-A foetus from a hypertensive pregnancy has more respiratory morbidity compared to the SGA-A foetus without maternal hypertensive disease due to lung damage caused by the maternal condition.

The purpose of this study was to evaluate a population of premature neonates (GA <34 weeks) with a birth weight <10th percentile (88) and to correlate Doppler-ultrasound findings and maternal hypertensive disease with neonatal respiratory outcome.

Methods

All neonates admitted to the neonatal intensive care unit (NICU) of the University Medical Centre Utrecht, the Netherlands are prospectively collected in a database. From this database we selected all neonates with birth weight <10th percentile (88) and GA <34 weeks born between 1st January 1999 and 31st December 2003. GA was calculated from the last menstrual period and early sonographic examination. We excluded infants with congenital, chromosomal or syndromal abnormalities.

The following data were recorded from the obstetric files: antenatal steroid treatment, maternal hypertensive disease, Doppler indices (pulsatility index (PI)) of the umbilical artery in the week before birth, multiple gestation, preterm premature rupture of membranes (PPROM), GA at delivery and route of delivery. Antenatal steroids were administered at the discretion of the attending obstetrician. Steroid treatment consisted of two doses of 12 mg of betamethasone (i.m.) 24 hours apart. Treatment was considered optimal in the group of infants whose mothers received two injections more than 24 hours before delivery. Treatment was considered incomplete if only 1 dose of steroids was administered before birth or if insufficient time had passed between administration of the second dose and birth (<24 hours). Incompletely treated infants were analyzed separately from the infants that received no or complete antenatal steroid treatment. Maternal hypertensive disease was defined as pregnancy induced hypertension (PIH), pre-eclampsia (PE) or HELLP syndrome (haemolysis, elevated liver enzymes, low platelets) (89). Doppler measurements were considered abnormal if the umbilical artery PI was >2 SD from the reference curve (90).

The following neonatal data was recorded: birth weight, sex, Apgar scores, umbilical cord arterial and venous pH, occurrence and grade of RDS, need for surfactant treatment and number of doses, ventilation, occurrence of chronic lung disease (CLD) and neonatal mortality. Neonates with clinical signs of RDS with oxygen requirement and typical findings on a chest X-ray were classified as having RDS (91). Surfactant treatment was given in case of signs of RDS on X-ray with need for mechanical ventilation and oxygen requirement >40% or mean airway pressure >10 cm H_20. CLD was defined as the need for extra oxygen on day 28 of life with chronic abnormalities on a chest X-ray and symptoms of respiratory distress. Postnatal steroids were given if ventilation continued for more than 1 week with an oxygen requirement >30%, despite treatment with diuretics and restriction of fluid intake. Neonatal mortality was defined as death within the first 28 days of life.

Statistical analysis was performed in the Statistical Package for the Social Sciences (SPSS) version 12.0.1 by independent t-tests for scale data and Chi-square tests for nominal

data. Fisher's exact test was used when the count was less than 5 in any one cell. Partial correlation was performed for scale data to correct for potential confounding factors. For nominal data, binary logistic regression was used for this purpose. Variables were considered to be potential confounders when the Chi-square test or independent t-test identified a significant difference. For all tests a value of $p<0.05$ was considered statistically significant.

The study was approved by the Institutional Review Board of the University Medical Centre Utrecht, the Netherlands.

Results

The study population consisted of 187 infants. Twenty-four percent (n=45) had normal Doppler examinations and constituted the SGA-N group. The 142 infants with signs of placental insufficiency (abnormal Doppler PI) were designated as being IUGR and constituted the SGA-A group (figure 1). Baseline characteristics of the two groups are shown in table 1. The HELLP subgroup contained the highest percentage of 'sick' women, since pregnancy was terminated (at least in part) for maternal reasons in 63-80% of cases.

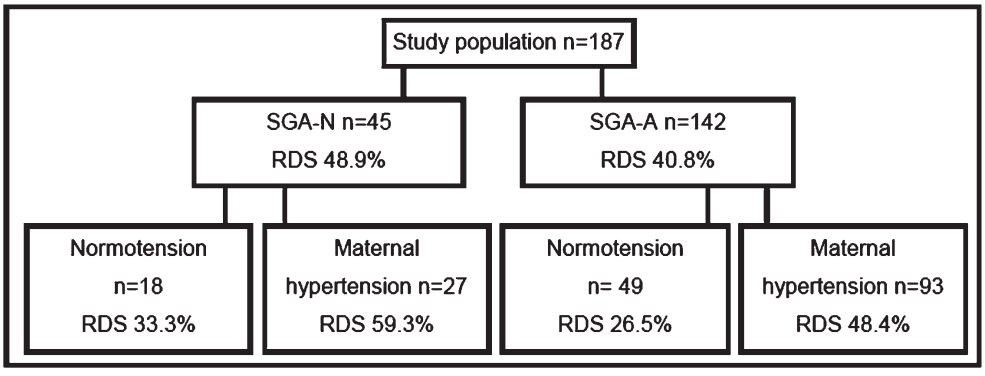

Figure 1 Study population
SGA-N= SGA foetus with normal Doppler examination of the umbilical artery
SGA-A= SGA foetus with abnormal Doppler examination of the umbilical artery
RDS= respiratory distress syndrome

The small for gestational age foetus: normal versus abnormal Doppler

The SGA foetuses with abnormal Doppler examination (SGA-A) showed a slightly better respiratory outcome compared to those with normal Doppler (table 1). However, no statistically significant difference in respiratory outcome was found between groups after correction for potentially confounding factors. There was a trend towards more neonatal death in the abnormal Doppler (SGA-A) group (adjusted odds ratio (OR) 4.9 (95% CI 0.8-28.9) p-value 0.08).

Table 1 Baseline characteristics: normal Doppler (SGA-N) versus abnormal Doppler (SGA-A).

	Normal Doppler (SGA-N)	Abnormal Doppler (SGA-A) *	Adjusted OR (95% CI) †	Corrected p-value ‡
Number of patients (n)	45	142	-	-
Complete steroid treatment %	77	80	-	-
Hypertensive disease % None	40	34	-	-
PIH	4	12		
PE	22	27		
HELLP	34	27		
PPROM %	24	3 §	-	-
Caesarean section %	80	99 §	-	-
Multiple gestation %	24	14	-	-
GA at delivery in weeks	29.9±2.0	30.5±1.8	-	-
Birth weight in grams	911±203	900±224	-	-
Birth weight <p2.3 %	27	49 ‖	-	-
Male sex %	60	54	-	-
Apgar <5 at 1' %	29	17	-	-
Apgar <6 at 5' %	7	1	-	-
Umbilical cord arterial pH	7.20±0.11	7.19±0.09	-	-
Umbilical cord venous pH	7.24±0.09	7.22±0.08	-	-
RDS %	49	41	0.6 (0.3-1.5)	0.28
RDS-grade ≥ 3 %	24	13	0.5 (0.2-1.3)	0.15
Surfactant %	36	26	0.8 (0.3-2.3)	0.69
Number of surfactant doses	2.6±1.2	2.4±1.4	-	0.67
CLD %	31	20	0.5 (0.2-1.3)	0.14
Postnatal steroids for CLD %	27	16	0.4 (0.1-1.2)	0.09
Neonatal death %	9	11	4.9 (0.8-28.9)	0.08
Ventilation %	53	43	0.7 (0.3-1.7)	0.38

Data presented as mean ± SD, unless stated otherwise.
* Independent t-test or Chi square test (Fisher's exact if count <5 in any one cell)
† Binary logistic regression ‡ Partial correlation or binary logistic regression
'Birth weight <p2.3'= birth weight below the 2.3rd percentile for gestational age
Significance of p-values **before** correction for potential confounders: ‖ p<0.01 § p<0.001

Maternal hypertension in the SGA-N foetus

If maternal hypertension was present, foetuses seemed to fare less well. However, no statistically significant differences in outcome were detected between SGA-N foetuses with or without maternal hypertensive disease (table 2), irrespective of adjustment for potential confounders.

Table 2 Normal Doppler (SGA-N): no maternal hypertensive disease versus maternal PIH/PE/HELLP.

	No hypertensive disease	PIH/PE/HELLP *	Adjusted OR (95% CI) †	Corrected p-value ‡
Number of patients (n)	18	27	-	-
Complete steroid treatment %	61	89	-	-
Hypertensive disease % None	100	0 §	-	-
PIH	0	7		
PE	0	37		
HELLP	0	56		
PPROM %	56	4 §	-	-
Caesarean section %	56	96 §	-	-
Multiple gestation %	50	7 ‖	-	-
GA at delivery in weeks	29.8±2.2	30.0±1.9	-	-
Birth weight in grams	920±195	906±212	-	-
Birth weight <p2.3 %	22	30	-	-
Male sex %	72	52	-	-
Apgar <5 at 1' %	33	26	-	-
Apgar <6 at 5' %	11	4	-	-
Umbilical cord arterial pH	7.20±0.11	7.19±0.11	-	-
Umbilical cord venous pH	7.26±0.10	7.22±0.08	-	-
RDS %	33	59	1.8 (0.3-11.8)	0.53
RDS-grade ≥ 3 %	22	26	1.1 (0.1-9.2)	0.93
Surfactant %	22	44	3.8 (0.4-34.4)	0.23
Number of surfactant doses	2.7±0.5	2.5±1.3	-	0.92
CLD %	22	37	4.0 (0.3-47.9)	0.28
Postnatal steroids for CLD %	17	33	-	0.99
Neonatal death %	17	4	0.9 (0.03-35.3)	0.98
Ventilation %	50	56	0.5 (0.07-3.4)	0.48

Data presented as mean ± SD, unless stated otherwise.
* Independent t-test or Chi square test (Fisher's exact if count <5 in any one cell)
† Binary logistic regression ‡ Partial correlation or binary logistic regression
'Birth weight <p2.3'= birth weight below the 2.3rd percentile for gestational age
Significance of p-values **before** correction for potential confounders: ‖ p<0.005 § p<0.001

Maternal hypertension in the SGA-A foetus

Within the abnormal Doppler examination-group (n=142), foetuses from hypertensive pregnancies had a poorer respiratory outcome (table 3). After correction for potentially confounding factors, foetuses in the maternal hypertension group still had a significantly higher chance of developing RDS (OR 2.7, 95% CI 1.0-7.1, p-value 0.04).

Table 3 Abnormal Doppler (SGA-A): no maternal hypertensive disease versus PIH/PE/HELLP.

	No hypertensive disease	PIH/PE/HELLP *	Adjusted OR (95% CI) †	Corrected p-value ‡
Number of patients (n)	49	93	-	-
Complete steroid treatment %	71	85	-	-
Hypertensive disease % None	100	0 §	-	-
PIH	0	18		
PE	0	41		
HELLP	0	41		
PPROM %	4	2	-	-
Caesarean section %	98	100	-	-
Multiple gestation %	31	5 §	-	-
GA at delivery in weeks	31.2±1.9	30.1±1.6 §	-	-
Birth weight in grams	940±247	880±209	-	-
Birth weight <p2.3 %	55	46	-	-
Male sex %	51	56	-	-
Apgar <5 at 1' %	16	17	-	-
Apgar <6 at 5' %	0	2	-	-
Umbilical cord arterial pH	7.20±0.09	7.18±0.09	-	-
Umbilical cord venous pH	7.22±0.09	7.22±0.08	-	-
RDS %	27	48 ‖	2.7 (1.0-7.1)	0.04
RDS-grade ≥ 3 %	4	18 ‖	4.0 (0.8-20.8)	0.10
Surfactant %	14	32 ‖	1.7 (0.5-6.0)	0.41
Number of surfactant doses	1.3±0.8	2.7±1.4 ¶	-	0.01
CLD %	12	25	1.5 (0.5-4.8)	0.46
Postnatal steroids for CLD %	8	20	2.1 (0.6-7.2)	0.27
Neonatal death %	10	12	0.8 (0.2-2.8)	0.74
Ventilation %	33	48	1.4 (0.5-3.6)	0.53

Data presented as mean ± SD, unless stated otherwise.
* Independent t-test or Chi square test (Fisher's exact if count <5 in any one cell)
† Binary logistic regression ‡ Partial correlation or binary logistic regression
'Birth weight <p2.3'= birth weight below the 2.3rd percentile for gestational age
Significance of p-values before correction for potential confounders: ‖ p<0.05 ¶ p<0.005 § p<0.001

Table 4 Abnormal Doppler (SGA-A): subdivisions.

	Normotension	PIH	PIH vs. normotension		PE	PE vs. normotension		HELLP	HELLP vs. normotension	
			OR 95%CI	p-value		OR 95%CI	p-value		OR 95%CI	p-value
Number of patients (n)	49	17	-		38	-		38		
Antenatal steroids %	71	77	-		89	-		84		
PPROM %	4	6	-		3	-		0		
Multiple gestation %	31	12	-		3	-		5		
Caesarean section %	98	100	-		100	-		100		
GA in weeks	31.2±1.9	29.9±2.1	-		30.4±1.4	-		30.0±1.6		
Birth weight in grams	940±247	824±230	-		899±217	-		885±193		
Birth weight <p2.3 %	55	47	-		37	-		55		
Male sex %	51	41	-		50	-		68		
Umbilical pH arterial	7.20±0.09	7.20±0.07	-		7.17±0.09	-		7.17±0.10		
venous	7.22±0.09	7.25±0.04			7.21±0.09			7.22±0.09		
RDS %	27	53	2.0(0.5-7.7)	0.30	26	0.8(0.3-2.5)	0.70	68	5.6(1.7-18.3)	0.004
RDS-grade ≥ 3 %	4	24	4.2(0.4-40)	0.21	5	1.0(0.1-8.4)	0.99	29	6.7(1.2-38.5)	0.03
Surfactant %	14	29	0.5(0.1-4.5)	0.55	16	0.7(0.2-3.1)	0.64	50	5.3(1.1-24.4)	0.04
CLD %	12	24	1.2(0.3-5.7)	0.82	13	0.7(0.2-2.8)	0.63	37	2.4(0.7-8.1)	0.16
Postnatal steroids%	8	18	1.5(0.3-8.4)	0.63	13	1.2(0.3-5.4)	0.78	29	2.7(0.7-10.2)	0.14
Neonatal death %	10	12	0.5(0.1-3.8)	0.51	13	1.4(0.3-6.2)	0.68	11	0.7(0.1-3.6)	0.68
Ventilation %	32	41	0.5(0.1-2.3)	0.40	40	1.1(0.4-3.3)	0.91	61	2.0(0.7-6.4)	0.22

We subdivided the maternal hypertension group to see if severity of the disease influenced the outcome (table 4). Outcome was poorest in the HELLP group as compared to the maternal normotensive group after correction for potential confounders. The OR for RDS was 5.6 (95% CI 1.7-18.3, p-value 0.004). The ORs for RDS-grade and the need for surfactant treatment were 6.7 (95% CI 1.2-38.5, p-value 0.03) and 5.3 (95% CI 1.1-24.4, p-value 0.04), respectively.

Table 5 Antenatal steroid treatment in total study population: complete versus no treatment

	No antenatal steroid treatment	Complete antenatal steroid treatment *	Adjusted OR (95% CI) †	Corrected p-value ‡
Number of patients (n)	19	146	-	-
Hypertensive disease % None	63	32 §	-	-
PIH	21	9		
PE	11	28		
HELLP	5	31		
Abnormal Doppler %	79	77	-	-
PPROM %	5	8	-	-
Caesarean section %	100	95	-	-
Multiple gestation %	11	19	-	-
GA at delivery in weeks	31.0±1.6	30.3±1.9	-	-
Birth weight in grams	903±213	899±222	-	-
Birth weight <p2.3 %	63	43	-	-
Male sex %	47	54	-	-
Apgar <5 at 1' %	11	22	-	-
Apgar <6 at 5' %	0	3	-	-
Umbilical cord arterial pH	7.16±0.13	7.19±0.09	-	-
RDS %	42	44	0.6(0.2-1.8)	0.39
RDS-grade ≥ 3 %	11	16	0.9(0.2-4.4)	0.86
Surfactant %	16	31	1.3(0.3-5.1)	0.69
Number of surfactant doses	3.0±2.0	2.4±1.3	-	
CLD %	32	23	0.4 (0.1-1.2)	0.09
Postnatal steroids for CLD %	21	18	0.4 (0.1-1.6)	0.21
Neonatal death %	16	10	0.6 (0.2-2.6)	0.52
Ventilation %	58	45	0.4(0.1-1.0)	0.06

Data presented as mean ± SD, unless stated otherwise.
* Independent t-test or Chi square test (Fisher's exact if count <5 in any one cell)
† Binary logistic regression ‡ Partial correlation or binary logistic regression
'Birth weight <p2.3'= birth weight below the 2.3rd percentile for gestational age
Significance of p-values **before** correction for potential confounders: § p<0.01

When we compared PIH/PE to HELLP syndrome, again neonatal respiratory outcome was significantly worse in the latter subgroup (data not shown). The adjusted ORs for RDS, the need for surfactant treatment and CLD were 3.9 (95% CI 1.6-9.6, p-value 0.003), 3.7 (95% CI 1.5-9.4, p-value 0.006) and 2.8 (95% CI 1.0-7.6, p-value 0.04), respectively.

Antenatal steroid treatment

Antenatal steroid treatment was complete in 146 patients. Respiratory outcome was not better in the treated group compared to the untreated treated group. No statistically significant differences were found (table 5). Within the SGA-A group 112 mothers were treated with antenatal steroids. Again, outcome was not significantly different between the treated and non-treated groups (data not shown).

Discussion

In this study we found no difference in respiratory outcome between SGA infants without or with abnormal Doppler waveform patterns in the umbilical artery. The latter group is likely to represent the true IUGR infant, with SGA due to placental insufficiency. Within the abnormal Doppler waveform group there were large differences in outcome in relation to the absence or presence of maternal hypertensive disease with poorest outcome in infants of women with HELLP syndrome.

The possibility of selection bias cannot be excluded in this study, because of its retrospective design. We feel, however, that our comparison of SGA-N and SGA-A foetuses gives a better estimate of neonatal respiratory outcome for the SGA-A foetus than comparisons made in previous retrospective studies. In the present study, classification of cases and controls was performed according to the principles for case-control studies (92). Cases and controls were selected from the same pool (e.g. same gestational age, same birth weight) with only 1 factor that determined whether the foetus was a case or control (e.g. results from Doppler ultrasound examination). Also, the cut-off point of 34 weeks GA makes the study population more homogenous. Furthermore, all data are from a single tertiary referral centre where the same criteria and the same protocols were used to diagnose and treat the various hypertensive disease states. Neonatal care management also did not differ substantially during the 5-year study period. Correction for potential confounders makes our results reliable. However, when variables are closely linked, controlling for potential confounding factors may reduce the apparent effect of the independent variable (Doppler results and maternal hypertensive disease). This could

lead to a lower contribution of the independent variable to outcome. We decided to control for confounding factors which have been proven to have an independent effect on morbidity and mortality. Summarizing, the present study was conducted properly with appropriate numbers in subgroups and therefore gives a reasonably valid view of respiratory outcome in this specific obstetric population. However, a prospective study will be necessary to validate our findings.

The similar outcome in SGA-N infants as compared to the SGA-A group indicates that SGA-A foetuses do not have accelerated pulmonary maturation. There are some publications that have assessed the relationship between Doppler waveform patterns of umbilical or uterine arteries with neonatal respiratory outcome in SGA infants (25-27). The differences with the present study mainly concern the wide gestational age range included (25-42 weeks). In a relatively small study (n=61) no relationship between (degree of) Doppler abnormality and RDS was found (27), whereas in two other publications a higher incidence of RDS was reported in the abnormal Doppler group (25;26). Unfortunately, in the large study by Soregaroli et al (26) no correction was performed for GA and birth weight, even though these variables varied considerably between Doppler-groups (GA 29 versus 34 and 37 weeks; birth weight 744 versus 1600 and 2120 grams in the reverse flow, abnormal PI and normal Doppler groups, respectively). None of these studies included the maternal condition in the analysis. The results of the present study, however, strongly suggest that underlying maternal hypertensive disease accounts for the different results.

When we take maternal hypertensive disease into account it becomes clear that SGA-A foetuses with maternal hypertensive disease do significantly worse than those with otherwise healthy mothers. This difference was exclusively explained by the maternal HELLP group (significantly higher RDS-incidence, -grade and need for surfactant treatment). Recently another group also found that HELLP-syndrome causes significantly more RDS in preterm infants (30). This study is different to ours in that neonatal outcomes of 21 infants born from pregnancies complicated by HELLP syndrome were compared to outcomes of 50 infants born preterm to normotensive mothers. Strict birth weight and/or gestational age inclusion criteria were not used which resulted in higher gestational age at delivery and birth weight (average GA 33 weeks; average birth weight 1500-2000 grams). In contrast, our study specifically studied SGA foetuses with or without abnormal Doppler ultrasound examination and with or without HELLP syndrome.

One may suggest that respiratory outcome is worse in infants from pregnancies complicated by HELLP syndrome due to an earlier delivery with a lower incidence of antenatal steroid treatment in cases with severe HELLP syndrome, but in the present study the difference in respiratory outcome remained significant after controlling for these potential confounders. A possible explanation could be that HELLP syndrome leads

to increased oxidative stress in the growth restricted foetus (93). In a study by Diedrich et al maternal markers of oxidative stress were significantly higher in HELLP syndrome than in pre-eclampsia ($p<0.01$) or normal pregnancy ($p<0.001$) (94). Oxidative stress could lead to lung damage and consequent detrimental neonatal respiratory outcome (28). Further research into the pathophysiological pathways leading to increased respiratory distress in neonates born to HELLP syndrome mothers and to quantify the extent of oxidative stress in the HELLP versus the PE foetus seems warranted.

Antenatal steroid treatment was not protective in this study population. Proof from other studies for the reduction of RDS in SGA neonates treated with antenatal corticosteroids is not conclusive. Bernstein et al showed that prenatal corticosteroid treatment resulted in a significantly lower risk of RDS and neonatal death in SGA neonates (84). The reduction in incidence of neonatal RDS, however, was smaller in SGA infants than in normally grown neonates. In contrast, four other groups (83;85;95;96) were not able to prove a beneficial effect of antenatal corticosteroids in SGA neonates on incidence of RDS. Antenatal corticosteroids did result in less intraventricular haemorrhage (IVH) in two of these studies (83;96). Schaap et al compared corticosteroid-treated IUGR neonates with non-treated IUGR-neonates. In all cases, IUGR was due to placental insufficiency. They were not able to find a reduction in RDS or severe IVH. However, there was a tendency towards lower mortality in the treated group (97). The growth restricted foetus is subjected to prolonged intra-uterine stress and the adrenal gland may already be stimulated (98). Therefore, there may be no additional effect of exogenous steroid administration in this particular group. Routine use of antenatal steroid treatment in SGA infants should be re-evaluated as the potential detrimental side effects of steroids on growth (99) are specifically unwanted in this already growth-restricted group.

Conclusion

SGA foetuses with abnormal Doppler examination have a similar neonatal respiratory outcome to those with normal Dopplers. Accelerated pulmonary maturation does not seem to be the case. SGA-A foetuses from mothers with HELLP syndrome have a significantly poorer neonatal respiratory outcome than SGA-A foetuses with otherwise healthy mothers. Possibly, oxidative stress in HELLP syndrome leads to lung damage rather than lung maturation. Antenatal steroid treatment did not result in less respiratory disease. It seems worthwhile to re-evaluate the routine use of corticosteroids for lung maturation in these infants as the potential detrimental side effects of steroids on growth are definitely unwanted in this already growth-restricted group.

3

LUNG MATURATION IN SMALL FOR GESTATIONAL AGE FOETUSES FROM PREGNANCIES COMPLICATED BY PLACENTAL INSUFFICIENCY OR MATERNAL HYPERTENSION

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Abstract

Background

Clinical studies suggest that respiratory outcome of infants born preterm may be influenced by placental insufficiency and haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome. If so, one could expect to see differences in lung maturation indices (lecithin/sphingomyelin (L/S) ratio and lamellar body count (LBC)) in the amniotic fluid. The present study investigates lung maturation indices of preterm small for gestational age (SGA) foetuses with or without abnormal Doppler ultrasound examination and with or without maternal hypertension/HELLP syndrome.

Methods

Retrospective cohort study of 76 neonates born in our centre between 1997 and 2003 with gestational age (GA) <34 weeks, birth weight <10th percentile for GA and available results from amniocentesis. All analyses were corrected for potential confounders.

Results

The L/S ratio was significantly higher in the abnormal Doppler group as compared to the normal Doppler group (p=0.02). The L/S ratio was significantly lower in hypertensive pregnancies as compared to normotensive pregnancies (p=0.02). Subdivision of the maternal hypertension group showed a significantly lower L/S ratio in the HELLP syndrome group as compared to the normotension group (p=0.04).

Conclusion

The L/S ratio of SGA foetuses is significantly higher in cases with presumed placental insufficiency and significantly lower when pregnancies are complicated by HELLP syndrome. These observations are in line with the hypothesis that placental insufficiency accelerates lung maturation and with recent reports of poorer respiratory outcome in infants from mothers with HELLP syndrome.

Introduction

Low foetal weight for gestational age is an important cause of neonatal morbidity and mortality (9;79;84). When foetal weight is below the 10th percentile for gestational age the foetus is designated as being small for gestational age (SGA). SGA may be caused by many factors including placental insufficiency which can be objectified by Doppler ultrasound examination (27). It has been hypothesised that placental insufficiency may accelerate pulmonary maturation through chronic intrauterine stress (24). However, to date no studies have been able to confirm this hypothesis (25-27;100). Contrary to expectation, two groups have reported a higher respiratory distress syndrome (RDS) incidence in infants with abnormal prenatal Doppler examination (25;26). Furthermore, recently we and others have shown that haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome (89) increases respiratory morbidity in preterm neonates (30;100), indicating impaired pulmonary development.

If lung development is influenced by placental insufficiency and HELLP syndrome, one would expect to see differences in lung maturation indices in the amniotic fluid. Previous research is inconclusive with the lecithin/sphingomyelin (L/S) ratio being unchanged by maternal hypertensive disease in some studies (101-103) and decreased in others (104;105).

To our knowledge, no research has been performed in preterm SGA infants to correlate the L/S ratio and lamellar body count (LBC) with Doppler ultrasound findings and maternal hypertensive disease. We hypothesise that:

1) preterm SGA foetuses with abnormal umbilical artery Doppler ultrasound examination will have a higher L/S ratio and LBC than SGA foetuses of the same gestational age (GA) with normal umbilical artery Doppler ultrasound examination

2) preterm SGA foetuses from mothers with HELLP syndrome will have a similar or lower L/S ratio and LBC compared to SGA foetuses of the same GA with normotensive mothers.

Methods

All neonates born in the Perinatal Centre of the University Medical Centre, Utrecht, the Netherlands, before 34 weeks of gestation are admitted to the neonatal intensive or high care unit and are prospectively collected in a database. From this database we selected all infants born between 1st January 1997 and 31st December 2003 with birth weight <10th percentile (88) and GA <34 weeks. GA was calculated from the

last menstrual period and early sonographic examination. For the present study we selected all cases in which amniocentesis had been performed for either foetal lung maturation determination or karyotyping. We excluded infants with major congenital, chromosomal or syndromal abnormalities.

The following data was recorded from the obstetric files: maternal age, parity, antenatal steroid treatment, Doppler indices (pulsatility index (PI)) of the umbilical artery in the week before birth, maternal hypertensive disease, multiple gestation, preterm premature rupture of membranes and GA at delivery. Antenatal steroid treatment consisted of two doses of 12 mg of betamethasone (i.m.) 24 hours apart. Treatment was considered optimal in the group of infants whose mothers received two injections more than 24 hours before amniocentesis. Steroids were administered at the discretion of the attending obstetrician. Doppler measurements were considered abnormal if the umbilical artery PI was >2 SD from the reference curve (90). Maternal hypertensive disease was defined as pregnancy induced hypertension (PIH), pre-eclampsia (PE) or HELLP syndrome (89). PIH was diagnosed if the following criteria were met: systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg from a GA of 20 weeks and onwards in women with previously normal blood pressure. PE was defined as a combination of PIH with proteinuria (≥ 300 mg/24 hours). The diagnosis HELLP syndrome was made when the following laboratory abnormalities were present: AST > 70 U/L, ALT >70 U/L, LDH > 600 U/L, platelet count < $100x10^9$/L and evidence of haemolysis. Recorded neonatal data included birth weight and sex.

Statistical analysis was performed in the Statistical Package for the Social Sciences (SPSS) version 12.0 by Chi-square tests for nominal data. Fisher's exact test was used when the expected count was less than 5 in any one cell. Normally distributed scale data are summarised as means ± SD and were analyzed by independent t-tests. Non-normally distributed scale data are summarised as median [range] and differences were analyzed by Mann Whitney U tests. Normalization was performed by taking the natural logarithm of the values. Next, ANCOVA analysis was used to reveal how L/S ratio and LBC levels (dependent variables) were influenced by the independent variables (Doppler ultrasound examination of the umbilical artery, maternal hypertensive disease). GA at amniocentesis and complete steroid treatment before amniocentesis were covariates in all these analyses. If any parameters differed significantly between groups at baseline, these were also added to the model as covariates. For all tests a p-value <0.05 was considered statistically significant.

Results

In total 76 infants fulfilled the study criteria. Baseline criteria of the study population are shown in table 1. In 62 cases amniocentesis was performed once, in the remaining cases amniocentesis was repeated. Data from the last amniocentesis before delivery was used in the analyses.

Table 1 Baseline characteristics

n	76
Maternal age (years)	31 [18-38]
Nulliparity %	75
Multiple gestation %	18
PPROM %	0
Maternal hypertensive disease %: - PIH - PE - HELLP	72 9 25 38
Abnormal Doppler %: 2-3 SD absent ED flow reversed ED flow	85 44 32 9
Complete steroids before amniocentesis %	85.5
GA at last amniocentesis (weeks)	30.1±1.8
GA in days at delivery (weeks)	30.7±1.8
Birth weight (grams)	946±221
Male sex %	49

PPROM: preterm premature rupture of membranes; PIH: pregnancy induced hypertension; PE: preeclampsia; HELLP: haemolysis, elevated liver enzymes, low platelets syndrome (9); 2-3 SD: 2-3 standard deviations from the reference curve (17); ED: end diastolic; GA: gestational age

Figure 1 shows that at each gestational age, the L/S ratio was higher in the abnormal Doppler group than in the normal Doppler group. This difference in L/S ratio was significant before and after correction for confounders (p=0.02) (table 2). No significant difference in LBC was found between groups.

When we compared maternal hypertension (including HELLP syndrome) to maternal normotension, it appeared that at most gestational ages hypertensive mothers had lower L/S ratios (figure 2). The L/S ratio was indeed significantly lower in hypertensive pregnancies as compared to normotensive pregnancies (p=0.006) (table 3). This difference remained significant after correction for gestational age at amniocentesis, multiple gestation, steroid treatment and foetal sex (p=0.02). Again, no significant difference in LBC was found between groups.

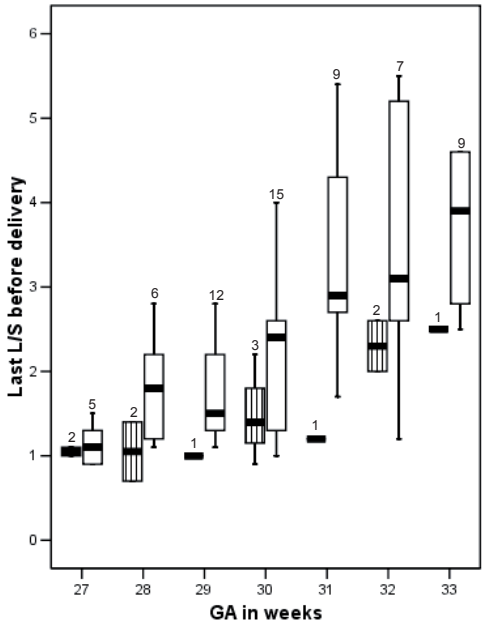

Figure 1 Normal versus abnormal umbilical artery Doppler ultrasound examination

▥ Normal Doppler

☐ Abnormal Doppler

The bars represent medians and quartiles. Numbers above bars represent sample sizes.

Table 2 Normal versus abnormal umbilical artery Doppler ultrasound examination

	Normal Doppler	Abnormal Doppler	*p*	Adj. *p**
N	12	64		
GA at last amniocentesis (weeks)	29.8±2.0	30.2±1.8	0.44	
Complete steroid treatment %	91	79	0.33	
L/S	1.3 [0.7-2.6]	2.5 [0.9-14.1]	0.003	0.02
LBC	8 [2-38]	15 [2-243]	0.08	0.12

See text for tests used for statistical analysis

* adjusted *p* value; GA: gestational age; L/S: lecithin/sphingomyelin ratio; LBC: lamellar body count

Table 3 also shows the L/S ratios after subdivision of the maternal hypertension group. The L/S ratio of the HELLP syndrome group was significantly lower than that of the normotensive group (p=0.005) and this difference remained significant after correction for confounders (p=0.04) (table 3).

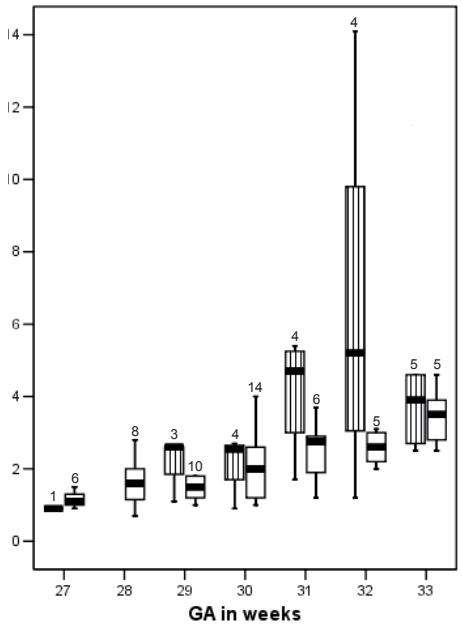

Figure 2 Maternal normotension versus maternal hypertension

▥ Maternal normotension

☐ Maternal hypertension.

The bars represent medians and quartiles; Numbers above bars represent sample sizes.

The difference in L/S ratio between the PE and normotensive group was significant before correction for confounders; however, after correction significance was lost. The relative risk (RR) for a low L/S ratio (L/S ratio<2.0) was 2.9 for the HELLP syndrome group as compared to the normotensive group (95% confidence interval (CI) 1.3-6.4) (table 3). The RR for HELLP syndrome versus PE was 1.8 with the lower limit of the 95% CI just above 1.0 (1.01-3.0). After correction for confounders, the LBC did not differ significantly between groups in any of the subgroup analyses.

Table 3 Maternal normotension versus maternal hypertension and subdivision

	Maternal normotension	Maternal hypertension	*p*	Adj. *p* *
n	21	55		
GA at amniocentesis (weeks)	31±1.7	29.8±1.8	0.008	
Complete steroids %	75	83	0.63	
L/S	2.7 [0.9-14.1]	1.8 [0.7-4.6]	0.006	0.02
LBC	15 [2-95]	10 [2-243]	0.21	0.72

	Maternal normotension	PIH	*p* †	Adj. *p* ‡	PE	*p* §	Adj. *p* ‖	HELLP	*p* ¶	Adj. *p* #
n	21	7			19			29		
GA at amniocentesis (weeks)	31±1.7	29.7±2.6	0.14		30.1±1.4	0.08		29.6±1.8	0.006	
Complete steroids %	75	86	0.77		82	0.38		83	0.80	
L/S	2.7 [0.9-14.1]	2.6 [1.2-4.0]	0.40	0.88	2.2 [0.7-4.6]	0.03	0.14	1.8 [0.9-3.7]	0.005	0.04
LBC	15 [2-95]	22 [20-49]	0.31	0.11	15 [2-243]	0.66	0.64	8 [2-98]	0.04	0.53
L/S<2.0 %	24	43	-	-	39	-	-	69	-	-
RR for L/S<2.0 (95% CI)	-	1.8 (0.6-5.7)	-	-	1.6 (0.6-4.3)	-	-	2.9(1.3-6.4)	-	-

See text for tests used for statistical analysis
† *p* value: PIH versus normotension
§ *p* value: PE versus normotension
¶ *p* value: HELLP versus normotension
* Adjusted *p* value: hypertension versus normotension
‡ adjusted *p* value: PIH versus normotension
‖ adjusted *p* value: PE versus normotension
adjusted *p* value: HELLP versus normotension

Discussion

The present study shows that the L/S ratio of SGA foetuses is significantly higher in pregnancies with abnormal umbilical artery blood flow velocity waveform patterns (placental insufficiency) and significantly lower in pregnancies complicated by maternal hypertension. Subdivision of the maternal hypertension group showed that low L/S ratios are particularly found in pregnancies complicated by HELLP syndrome. Finally, the LBC did not differ significantly between groups in any of the analyses.

This discrepancy between the L/S ratio and LBC can in part be explained. First of all, in the Doppler analysis a higher (non-significant) LBC was seen in the abnormal Doppler group, which is in line with the L/S results. Possibly groups were too small to detect significant differences in the LBC. Furthermore, the amount of amniotic fluid may affect

the LBC, but an effect on the L/S ratio seems less likely, since dilution or concentration affects both lecithin and sphingomyelin to the same extent (106-108).
An important limitation of the study is its retrospective design which may have resulted in selection bias. By including all neonates that fulfilled the study criteria, we aimed to keep this risk as small as possible. However, it has to be noted that only infants with results from amniocentesis were eligible for the study. This means only babies/mothers who were stable enough to undergo amniocentesis and receive steroids to enhance pulmonary maturation were included. Pregnancies that did not have time to undergo amniocentesis for maternal or foetal reasons will have been eliminated from the study group. Another limitation is the small number of patients included in the study. This prevented us from analyzing the data per gestational age week. A large prospective study may provide valuable gestational age dependent information and may validate the present results.
The results from the present study regarding placental insufficiency differ from earlier studies. No previous study has been able to confirm the hypothesis that placental insufficiency causes accelerated pulmonary maturation (25-27;100). From the present study it appears that L/S ratios are higher in the abnormal Doppler group, indicating that foetal lung maturation is enhanced with placental insufficiency. This difference may be explained by the design of the present study as compared to previous studies. We studied biochemical parameters of foetal lung maturity and not clinical respiratory outcome. Foetal lung maturation indices are measured objectively and on an absolute scale whereas clinical respiratory outcome is measured subjectively.
From recent clinical studies we had reason to believe that HELLP syndrome may cause respiratory morbidity (30;100). Interestingly, in line with these reports the present study shows that maternal hypertensive disease and especially HELLP syndrome are associated with a lower L/S ratio. Two previous studies also found that L/S ratios were lower in pregnancies complicated by maternal hypertension (104;105). In contrast, three other studies found no difference in L/S ratios between hypertensive and normotensive pregnancies (101-103). Importantly, none of these groups studied the effect of maternal hypertension in preterm SGA foetuses (GA <34 weeks, birth weight <p10 for GA). The present study shows that within the SGA population, HELLP syndrome in particular is associated with lower L/S ratios. Possibly, increased oxidative stress in mothers with HELLP syndrome leads to increased formation of free radicals in the foetus. Oxidative stress has been shown to be increased in both PE and HELLP syndrome (93), however, clinical disease severity has been shown to play a role in the amount of oxidative stress measured (94;109) with a significantly higher degree of oxidative stress in pregnancies complicated by HELLP syndrome as compared to those complicated by PE (94). Some

studies have found that maternal oxidative stress causes placental changes (110) with increased oxidative stress in cord blood directly after birth (111). Results from a recent study by our group show that oxidative stress levels in plasma and exhaled breath are higher in preterm neonates from mothers with HELLP syndrome as compared to those from mothers with PE within 12 hours after birth (112). Oxidative stress can cause direct lung tissue damage, surfactant inactivation and inhibition of surfactant metabolism in the preterm infant (28). It is unclear, however, how maternal oxidative stress causes foetal/neonatal oxidative stress. Placental passage, maternal placental interaction or placental/foetal responses to the maternal changes may be the effector mechanism. Further research to elucidate these mechanisms is required.

4

OXIDATIVE STRESS AND PROINFLAMMATORY CYTOKINE LEVELS ARE INCREASED IN PREMATURE NEONATES OF PREECLAMPTIC MOTHERS WITH HELLP SYNDROME

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Abstract

Background

Respiratory distress syndrome (RDS) incidence is increased in infants of pre-eclamptic mothers with haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome. RDS and HELLP syndrome have been associated with oxidative stress and inflammatory processes. We hypothesise that end-tidal carbon monoxide corrected for inhaled CO (ETCOc), malondialdehyde (MDA) (markers of oxidative stress) and pro-inflammatory cytokine (Il-6, Il-8) production are higher in infants of pre-eclamptic mothers with HELLP syndrome than in those of pre-eclamptic mothers without HELLP syndrome.

Methods

Prospective study of 36 infants of pre-eclamptic mothers (GA<32 weeks) admitted to the NICU. ETCOc was measured at 0-12, 48-72 and 168h postnatally using the CO-Stat™ End-Tidal Breath Analyzer. Simultaneously, blood was sampled for MDA, Il-8 and Il-6.

Results

At 0-12h, ETCOc, MDA and Il-8 values (median [range]) were significantly higher in HELLP infants than in infants from pre-eclamptic mothers without HELLP (ETCOc 2.2 [1.5-3.9] vs. 1.8 [0.5-2.9] ppm; MDA 2.3 [1.3-4.1] vs. 1.5 [0.4-3.1] umol/L; Il-8 145 [24-606] vs. 62 [26-397] pg/mL; all $p<0.05$). MDA remained significantly higher during the first 168h of life (2.3 [0.8-5.8] vs. 1.1 [0.8-3.7] umol/L, $p=0.02$).

Conclusion

Oxidative stress and proinflammatory cytokine levels are increased in infants of pre-eclamptic mothers with HELLP syndrome. These processes may cause inactivation of surfactant explaining the increased RDS incidence in these infants.

Introduction

Respiratory distress syndrome (RDS) incidence is increased in infants of pre-eclamptic mothers with haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome (89) possibly due to lung damage caused by the maternal condition (30;100). RDS has been associated with oxidative stress and inflammatory processes (28;100;113-115), as have pre-eclampsia (PE) and HELLP syndrome (93;94;116;117). One study showed that maternal parameters of oxidative stress were significantly higher in women with HELLP syndrome as compared to women with PE (94).

Carbon monoxide (CO) is primarily produced by oxidative degradation of heme and diffuses from the blood to alveolar air. CO production is increased during oxidative stress and inflammation via lipid peroxidative processes, with a subsequent increase of CO in exhaled breath (118;119). Amongst the most common direct markers of oxidative stress is plasma malondialdehyde (MDA) which serves to quantify the oxidative damage of lipids (120). Inflammatory activity can be measured through determination of pro-inflammatory cytokines (e.g. interleukin (Il)-6 and Il-8).

The present study investigated levels of end-tidal CO corrected for inhaled CO (ETCOc) and plasma MDA, Il-6 and Il-8 in infants of women with pre-eclampsia or HELLP syndrome. We hypothesise that levels of ETCOc, MDA, Il-6 and Il-8 will be higher in infants of mothers with HELLP syndrome.

Methods

All infants of mothers with pre-eclampsia or HELLP syndrome (gestational age (GA) < 32 weeks) admitted to the Neonatal Intensive Care Unit (NICU) between January 1st and December 31st 2003 were included in this study after obtaining parental informed consent. Neonatal and obstetrical data were collected prospectively.

Pre-eclampsia was defined as an increased diastolic blood pressure (>90mm Hg) or increases of diastolic blood pressure of 15mm Hg over baseline values with proteinuria (> 300mg/24 hours) on urinalysis. The diagnosis HELLP syndrome was made when the following laboratory abnormalities were present: AST > 70 U/L, ALT >70 U/L, LDH > 600 U/L, platelet count < $100x10^9$/L and evidence of haemolysis. Maternal infection was diagnosed when maternal fever (>38.5° C), elevated C-reactive protein or positive blood cultures were present.

ETCOc was measured at 0 to 12, 48 to 72 and 168 h postnatally using the CO-Stat™ End-Tidal Breath Analyzer (Natus Medical Inc., San Carlos, CA, USA). Simultaneously,

blood was sampled for MDA, Il-6 and Il-8. The method to measure ETCOc has been described previously (113;121;122). Briefly, this non-invasive bedside instrument uses electrochemical sensors for measurement of CO, and an infrared optical bench for measurement of end-tidal carbon dioxide. Using CO-Stat™, ETCOc could be measured reliably and reproducibly in these tiny ventilated infants (113). Measurements were performed in duplicate and the mean value was reported.

MDA in plasma was measured using a high-pressure liquid chromatography method after mixing equal volumes of plasma and 10% (w/v) metaphosphorix acid containing 2 mM Desferal and storage at -80 degrees Celsius (123).

Cytokine levels were determined by enzyme-linked immosorbent assay (Pelikine; CLB, Amsterdam, the Netherlands). Detection limits were 2.5 pg/mL for Il-6 and Il-8.

Infants with clinical signs of RDS with oxygen requirement and typical findings on a chest X-ray were classified as having RDS (91). If RDS-grade I or II (according to Giedion (91)) was present on chest X-ray and no artificial surfactant replacement treatment was needed, RDS was classified as 'moderate'. 'Severe' RDS was classified as Giedion grading III or IV and the need for surfactant replacement therapy. The decision to administer surfactant was made by the attending neonatologist according to a defined protocol. Surfactant was administered to neonates with GA <29 weeks, if ventilation is needed for treatment of RDS within 1 hour after birth and, to infants with GA >29 weeks, if FiO2 >0.4 and mean airway pressure >10 cm H20. Neonatal infection was diagnosed if children had positive blood cultures or antibiotic treatment for clinical sepsis in the first 7 days of life.

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) version 12.0. Data are summarised as median with [range]. Differences between groups were assessed by Mann Whitney U test. Differences in nominal variables were assessed by Chi-square test; Fisher's exact test was used when the count was less than 5 in any one cell. Statistical significance was assumed for all tests when $p< 0.05$.

The study was approved by the Institutional Review Board of the University Medical Centre Utrecht, the Netherlands. Informed consent was obtained from all parents.

Results

Thirty-six infants of pre-eclamptic mothers were included, of which 19 had mothers with HELLP syndrome. The clinical characteristics of the studied infants are shown in table 1. There were no differences between groups in birth weight, gestational age, Apgar scores or gender. In the HELLP group, significantly more infants received antenatal

glucocorticosteroids (p<0.05). Rate and severity of RDS were also higher in the HELLP syndrome group, but these differences did not reach significance. Furthermore, infants in the HELLP group were dependent on ventilation for a longer period of time (p<0.05).

Table 1 Characteristics of the study population

	Pre-eclampsia n = 17	HELLP syndrome n = 19
Birth weight (g)	930 [620-2100]	970 [685-1700]
Gestational age (wks)	30.4 [27.2-33.4]	30.1 [26.1-32]
SGA (n)	7	13
Apgar score (at 5 min)	9 [3-10]	8 [6-10]
Male sex (n)	8	12
Antenatal steroids (n)	11	18 *
Ventilation (n)	8	10
SIMV	5	3
HFO	3	7
Days on ventilation	5 [2-26]	12.5 [2-33] *
RDS (n)	8	10
Severe RDS (n)	2	6
Maternal infection (n)	0	0
Neonatal infection (n)	0	0
Placental weight <10th percentile (n)	8	15
Placental infarction (n)	10	9

Data presented as median [range], unless stated otherwise. See text for tests used for statistical analysis.
*p<0.05 vs. pre-eclampsia
HELLP: haemolysis, elevated liver enzymes, low platelets
SGA: small for gestational age (birth weight below 10th percentile for gestational age (88))
SIMV: synchronised intermittent mandatory ventilation; HFO: high frequency oscillation
RDS: respiratory distress syndrome

Figure 1A shows that the ETCOc values were significantly higher in the HELLP group at 0-12h as compared to the pre-eclamptic group without HELLP syndrome (2.2 [1.5-3.9] vs. 1.8 [0.5-2.9]; p<0.02). During the first 7 days of life ETCOc decreased in the HELLP group, however, values remained higher than those of the PE group during the first 72 hours of life (2.2 [0.8-3.7] vs. 1.7 [1.1-2.6].

Figure 1B shows that plasma MDA was also significantly higher at 0-12h (2.3 [1.3-4.1] vs. 1.5 [0.4-3.1]; p<0.01), increased over time in the HELLP group and at 168h of life values remained significantly higher in the HELLP group (2.3 [0.8-5.8] vs. 1.1 [0.8-3.7]; p<0.02).

MDA and ETCO only showed a correlation at 168 h of life (R=0.42 p<0.05).

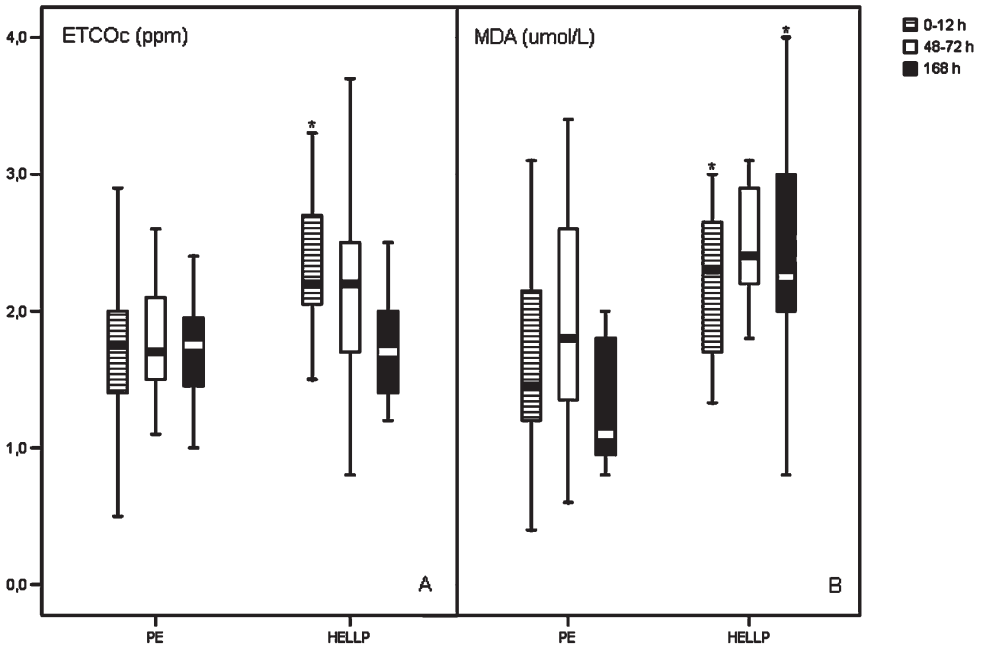

Figure 1 ETCOc (A) and MDA (B) values measured at three time points (0-12, 48-72 and 168h postnatally) in the first seven days of life
* p< 0.05 versus PE ppm: parts per million

Figure 2 shows the plasma cytokine values of the 2 groups at the various time points. Il-8 values were significantly higher at 0-12h (145 [24-606] vs. 62 [26-397]; $p<0.05$) (figure 2A). Il-6 values tended to be higher in the HELLP group, but never reached statistical significance (figure 2B). The correlation between Il-6 and Il-8 was significant within the first 72 h of life (R=0.52, p 0.001 and R=0.83, p 0.01 at 0-12 h and at 48-72 h, respectively).

We also analyzed ETCO, MDA, Il-6 and Il-8 according to absence or presence of RDS and found that these markers were higher in the RDS group at most time points. Significant differences were seen at 0-12 and 48-72 h for Il-6 and Il-8 ($p<0.01$).

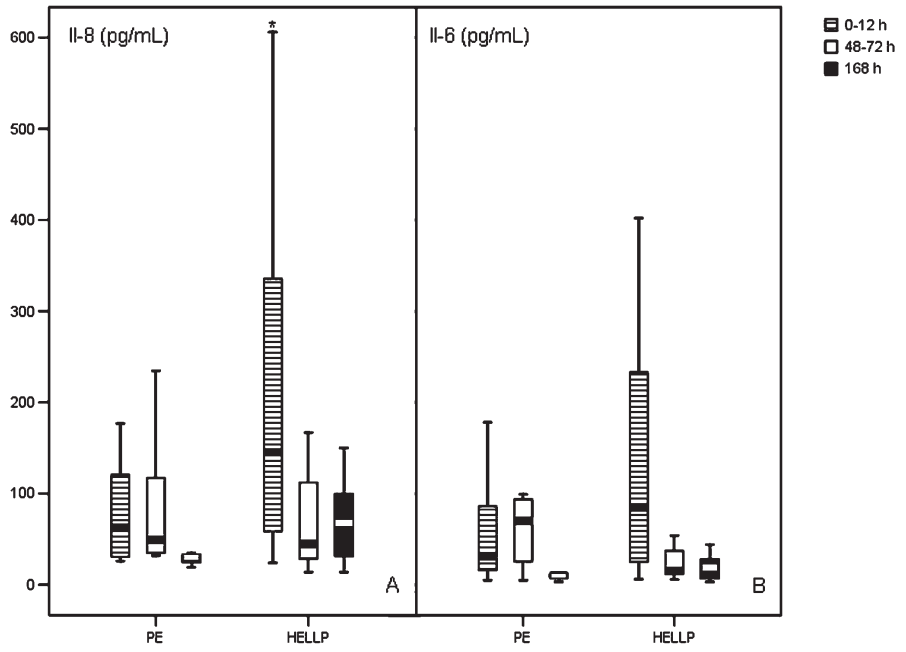

Figure 2 Il-8 (A) and Il-6 (B) values measured at three time points (0-12, 48-72 and 168h postnatally) in the first seven days of life
* p< 0.05 versus PE

Discussion

Oxidative stress and proinflammatory cytokine levels are increased in premature infants from mothers with HELLP syndrome as compared to those from mothers with pre-eclampsia.

Although severe RDS was indeed 2.5 times more frequent in the HELLP group (n=6 versus n=2) this difference was not quite significant, possibly due to the small number of infants. However, a previous study showed that higher ETCOc values were seen in children with RDS (113). Furthermore, a recent study by our group in a larger patient cohort showed that RDS incidence is significantly higher in infants of mothers with HELLP syndrome (100). In the present study, significantly more children in the HELLP group received antenatal steroid treatment. Antenatal steroids are known to promote a better respiratory outcome (33). However, contrary to expectation, rate of severe RDS was higher in the HELLP group as compared to the PE group (non-significant difference) and certainly not lower. Also, ventilation requirements were higher in infants of the

HELLP syndrome group, indicating worse respiratory outcome.
MDA and ETCO values did not correlate within the first 72 h of life, contrary to our expectation. This may indicate that MDA and ETCO are not elevated by the same processes. CO is not only produced by lipid peroxidation, but also by degradation of red blood cells which occurs in particular during the first days of life, while MDA is a product of lipid peroxidation. This may explain the lack of correlation in the first 72 hours of life and the positive correlation later, on postnatal day 7.
RDS is a clinical syndrome of multifactorial aetiology with the most important factor being surfactant deficiency. Various authors have suggested that oxidative stress and inflammation may also play important roles by causing lung tissue damage of the alveolar type II cell, inhibiting surfactant metabolism and causing surfactant inactivation (28;114;115;124;125). Since oxidative stress and proinflammatory cytokine levels were higher in neonates from the HELLP group, one could postulate that maternal HELLP syndrome promotes foetal and neonatal lipid peroxidation and pro-inflammatory activity. However, it is unclear how maternal oxidative stress causes neonatal oxidative stress. It can be speculated that placental passage, maternal placental interaction or placental/foetal responses to the maternal changes may be the effector mechanism. Some studies have indeed found that maternal oxidative stress causes placental changes (110) with increased oxidative stress in cord blood directly after birth (111). Further investigation into pathophysiological pathways leading to increased oxidative stress and inflammation in the HELLP syndrome neonate is necessary.
In conclusion, oxidative stress and proinflammatory cytokines are increased in premature infants of mothers with HELLP syndrome. These processes may cause lung tissue damage and inactivation of surfactant and thus may explain the increased RDS incidence found in infants of mothers with HELLP syndrome.

CHARACTERISTIC PLACENTAL PATHOLOGY IN EARLY-ONSET INTRAUTERINE GROWTH RESTRICTION WITH OR WITHOUT MATERNAL HYPERTENSIVE DISEASE

5

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Abstract

Background

Abnormal placental development is thought to underlie the pathogenesis of early-onset intrauterine growth restriction (IUGR), pre-eclampsia (PE) and HELLP syndrome. Little research has been performed to study differences in placental pathology between these various conditions.

Objective

The aim of the present study was to provide an overview of pathological differences in placentae from early-onset IUGR pregnancies with or without PE or HELLP syndrome. As a secondary outcome, the effect of increasingly abnormal blood velocity waveform patterns in the umbilical artery on placental pathology was evaluated.

Methods

Placentae from 164 neonates born with gestational age (GA) <34 weeks, birth weight <10th percentile for GA and abnormal Doppler of the umbilical artery (UA) were studied. UA end-diastolic (ED) flow was classified as being present (PED) or absent/reverse (ARED). In 66 cases the pregnancy was complicated by HELLP syndrome and in 58 cases by PE.

Results

Chronic inflammation of foetal membranes (p <0.05) and syncytial knotting (p <0.05) were more common in women with concomitant PE/HELLP syndrome as compared to women without maternal hypertensive disease. More positive nitrotyrosine staining (p <0.05) and less positive caspase-3 staining (p <0.05) of trophoblast nuclei was present in HELLP syndrome as compared to PE. Increased numbers of nucleated red blood cells (p <0.001) and distal villous hypoplasia (p <0.05) were present more often in ARED as compared to PED UA flow.

Conclusion

Increasingly abnormal UA flow is associated with increased presence of pathologic features characteristic of chronic hypoxia. Chronic inflammation of foetal membranes in PE and HELLP syndrome reflects activation of the maternal immune system. HELLP syndrome is associated with more placental oxidative stress and less apoptosis as compared to PE. Increased oxidative stress may cause a switch from apoptotic to necrotic trophoblast shedding. The latter is currently being studied.

Introduction

Abnormal placental development has been thought to underlie most cases of early-onset intrauterine growth restriction (IUGR) and pre-eclampsia (PE); two disorders that frequently, but not always, occur concomitantly (23;126). The maternal manifestations of these disorders, however, are different (23) and the pathophysiological differences remain to be elucidated. Haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome (89) is thought to be an extreme variant of severe PE with an unknown cause (127), generally considered as a potentially life-threatening condition, prompting immediate delivery.

Little research has been performed to compare features of placental pathology between these various disease states and more specifically between early-onset PE and HELLP syndrome (128;129). In the present study, a well-defined group of placentae from pregnancies complicated by early-onset IUGR resulting in delivery before 34 weeks of gestation were examined to detect differences in placental pathology between these conditions. The aim of the present study was to identify hallmark features of placental pathology that might discriminate between early-onset IUGR with or without concomitant maternal hypertensive disease. The effect of increasingly abnormal blood velocity waveform patterns in the umbilical artery on placental pathology was evaluated as a secondary outcome.

Methods

From a database containing data on all children admitted to the neonatal care unit at the University Medical Centre Utrecht, the Netherlands, we identified 185 preterm SGA neonates (gestational age (GA) < 34 weeks and birth weight <10th percentile for GA (88)) born between 1st January 1997 en 31st December 2004. From these neonates we selected foetuses with a diagnosis of intrauterine growth restriction (IUGR) due to placental insufficiency based on abnormal Doppler ultrasound examination of the umbilical artery (UA) (>2 standard deviations from the reference curve (90)) (n=164). UA end-diastolic (ED) flow was classified as being present (PED) or absent/reverse (ARED). Cases with maternal infection, vaginal delivery, preterm premature rupture of membranes, chromosomal abnormalities, foetal deaths, twins/triplets and infants with major congenital anomalies were excluded. PE was defined as a combination of systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg from a GA of 20 weeks onwards in women with previously normal blood pressure and proteinuria

(≥ 300 mg/24 hours) according to criteria of the International Society for the Study of Hypertension in Pregnancy (130). HELLP syndrome was diagnosed when the following laboratory abnormalities were present: AST > 70 U/L, ALT >70 U/L, LDH > 600 U/L, platelet count < $100x10^9$/L and evidence of haemolysis (131). We further defined three distinct study groups: IUGR with normotension (1; 40 cases), IUGR with PE (2; 58 cases) and IUGR with HELLP syndrome (3; 66 cases).

Histology

All placentae from complicated pregnancies were examined histologically. Placentae were weighed (without umbilical cord and membranes) and the percentage of infarction was determined. Subsequently, one sample of the umbilical cord and foetal membranes and at least two samples of macroscopically normal placental tissue were dissected from the central part of the placenta for routine histological examination. All samples were embedded in paraffin and stained with haematoxylin and eosin by standard histological procedures. For the present study, archived placental slides were reviewed by a perinatal pathologist (PN), blinded to the clinical outcome, according to a research protocol for the following histopathological aspects: chorioamnionitis, funiculitis, villitis, chronic inflammation of membranes, necrosis of membranes, nucleated red blood cells (NRBC), syncytial trophoblast knotting, maturation of placental parenchyma, foetal thrombosis and distal villous hypoplasia. Additionally, the ratio between infant birth weight and placental weight (foetoplacental weight ratio) was calculated. Definitions for placental findings are summarised in table 1 (adapted from Smulian et al (128)).
Because certain parameters differ with increasing gestational age (e.g. maturation of placental parenchyma progresses during gestation (132)) the pathologist was blinded to all clinical data except to GA at delivery. Histopathological aspects (as defined in table 1) were assessed as normal or absent, moderately increased or severely increased for GA on a scale of 0 to 2.

Immunohistology

For immunohistological staining, additional tissue sections were cut at 4 μm and mounted on coated slides from randomly selected placentae from all groups (1-13 cases; 2-14 cases and 3-15 cases). To study nitrotyrosine (NT) (a marker of oxidative stress), sections were stained with rabbit anti-human NT antibody (Upstate Biotechnology, Lake Placid, NY). Apoptosis was studied using a purified rabbit anti-human active caspase-3 monoclonal antibody (BD Pharmingen™, BD Biosciences, Europe). For all staining, visualization was performed using Powervision-HRP (ImmunoLogic, The Netherlands) and diaminobenzidine followed by counterstaining with haematoxylin. Positive immunohistological staining for NT and caspase was scored for various placental

components using semiquantitative analysis: no staining, weak staining, moderate staining and severe staining. Samples with weak, moderate or severe staining were considered positive in further analyses.

Table 1 Definitions for gross and histopathological placental findings (adapted from Smulian et al (128))

Placental finding	Definition
Placental weight	Weight of the placenta measured without umbilical cord and membranes
Fetoplacental weight ratio	The ratio of foetal to placental weight
Infarction	Presence of infarction in the placental disc measured as a percentage of total placental volume
Chorioamnionitis	Neutrophilic infiltration in the membranes or basal plate. Classification: A: Neutrophils in membranes or basal plate (mild) B: Inflammatory infiltrate in membranes and basal plate (moderate) C: see B with microabscesses in the basal plate (severe)
Funiculitis	Neutrophilic infiltration of the vessel wall of: A: the umbilical vein (mild) B: the umbilical vein and one artery (moderate) C: all three vessels of the umbilical cord (severe)
Villitis	Lymphocytic (and mononuclear) cells infiltrating: A: one cluster of 5 or more chorionic villi in 1 slide (mild) B: one cluster of 5 or more chorionic villi in 2 to 3 slides (moderate) C: at least 2 clusters of 5 or more chorionic villi in at least 3 slides (severe)
Chronic inflammation of the membranes	Lymphocytic infiltration of the membranes
Membrane necrosis	Presence of necrosis in the membranes
Elevated NRBC	Elevated levels of circulating nucleated erythrocytes in umbilical artery/vein
Increased syncytial knotting	Syncytial nuclei forming a multinucleated protrusion from the villous surface of terminal villi
Villous maturation (increased)	Maturation of chorionic villi in the foetal lobule matched with gestational age: appropriate or more advanced than stated age
Foetal thrombosis	Thrombosis of blood vessels in chorionic stem villi diagnosed by the presence of clusters of avascular terminal villi
Distal villous hypoplasia	Villous pattern characterised by slender, elongated, poorly branched and poorly capillarised (133)

Statistical analysis

Differences between groups were analyzed in the Statistical Package for the Social Sciences (SPSS) version 15.0 by chi square test (Fisher's exact test if the expected count was < 5) or Mann-Whitney U test, where appropriate.

Results

Placental pathology was observed in a majority of placentae from pregnancies complicated by early-onset IUGR, PE and HELLP syndrome (tables 2 and 3).

Table 2 Placental histological findings in IUGR placentae according to end-diastolic flow and maternal hypertensive disease

	Positive ED flow	Absent/reverse ED flow	Maternal normotension	PE	HELLP syndrome
Number of patients	82	82	40	58	66
AD (weeks)	30 5/7 [26 6/7-33 6/7]	30 1/7 [26-33 2/7]	31 1/7 [27-33 6/7]	30 3/7 [26-33 6/7] *	30 1/7 [26 6/7-33 4/7] †
Birth weight (grams)	900 [440-1440]	842 [450-1470]	830 [480-1470]	874 [455-1400]	900 [440-1440]
Placental weight (grams)	200 [106-400]	179 [90-326] **	180 [90-326]	197 [94-320]	188 [106-400]
Fetoplacental weight (ratio)	4.8 [1.5-7.2]	4.7 [2.8-9.9]	4.8 [1.9-8.3]	4.7 [2.7-9.9]	4.8 [1.5-7.2]
Infarction present	82	81	73	91 *	75 §
Chorioamnionitis	0	2	5	0	0
Funiculitis	0	2	5	0	0
Villitis	22	21	22	24	17
Chronic inflammation	33	39	19	40 *	42 ‡
Membrane necrosis	21	20	17	28	16
Elevated NRBC	88	90	93	91	83
Severely elevated NRBC	9	31 **	20	24	15
Increased knotting	94	92	81	98 ^	91 ‡
Increased maturation	99	98	93	100	100
Foetal thrombosis	31	34	42	29	29
Distal villous hypoplasia	27	44 ^^	37	33	38

All values represent percentages unless stated otherwise.
^^ $p < 0.05$ absent/reverse vs. positive ED flow
** $p < 0.001$ absent/reverse vs. positive ED flow
* $p < 0.05$ PE vs. normotension
^ $p < 0.01$ PE vs. normotension
‡ $p < 0.05$ HELLP syndrome vs. normotension
† $p < 0.01$ HELLP syndrome versus normotension
§ $p < 0.05$ PE vs. HELLP syndrome

Histology

First of all, placental features were studied according to the presence or absence of concomitant maternal hypertensive disease (table 2). Chronic inflammation of

the membranes and syncytial knotting were significantly increased in PE and HELLP syndrome as compared to maternal normotension. Placental infarction was observed more often in pregnancies complicated by PE as compared to normotension or HELLP syndrome (91 vs. 73-75%, p <0.05).

Secondly, severely elevated NRBC and distal villous hypoplasia were present significantly more frequently in placentae with ARED as compared to PED UA flow (31 vs. 9%, p<0.001 and 44 vs. 27%, p <0.05; respectively). Furthermore, placental weight was significantly lower in the former group (179 vs. 200 grams, p <0.001) (table 2).

Caspase-3 staining

Significantly more positive caspase-3 staining of trophoblast nuclei in fibrin was found in PE placentae as compared to HELLP syndrome (p <0.05) and in placentae with ARED as compared to PED UA flow (p <0.01) (table 3).

Table 3 Immunohistology in IUGR placentae according to end-diastolic flow and maternal hypertensive disease

	Positive ED flow	Absent/reverse ED flow	PE	HELLP syndrome
Number of patients	7	35	14	15
NT Trophoblast nuclei	33	29	8	55 ^
NT Stromal nuclei	80	90	100	82
NT Endothelium	80	87	100	73
NT Smooth muscle cell	80	90	100	73
NT Foetal blood	80	87	92	91
NT Maternal blood	100	87	100	91
Caspase Trophoblast nuclei	29	60	71	47
Caspase Fibrin	43	91 *	100	67 ^
Caspase Stromal nuclei	14	6	0	7
Caspase Knots	0	20	29	13

All values represent percentages
NT: nitrotyrosine
* p < 0.01 absent/reverse vs. positive ED flow
^ p < 0.05 PE vs. HELLP syndrome

Nitrotyrosine staining

For nitrotyrosine, positive NT staining of trophoblast nuclei was observed significantly more often in HELLP syndrome as compared to PE (55% vs. 8%, p <0.05)(table 3).

Discussion

The aim of the present study was to provide more insight into pathological differences in placentae from early-onset IUGR pregnancies with or without PE or HELLP syndrome. The main finding of this study is that characteristic placental pathology consistent with chronic hypoxia, vascular compromise, necrosis, inflammation and oxidative stress, can be observed in a majority of placentae from pregnancies complicated by early-onset IUGR, PE and HELLP syndrome.

Previous studies on placental histology of these conditions have reported heterogeneous features (128;129;133-139). This is possibly due to use of dissimilar definitions for IUGR (133-135), inclusion of small numbers of placentae (133;137;140) and inclusion of wide gestational age ranges (128;129;135-137). Importantly, it has been shown that certain placental parameters differ across gestation (132) and recently Moldenhauer et al (136) have confirmed that the frequency and severity of placental findings in PE are GA dependent, suggesting that strict GA inclusion criteria should be used when studying placentae.

The present study is unique as it was large, only included placentae from women who delivered an IUGR infant before 34 weeks of gestation and used Doppler ultrasound indices to diagnose IUGR, but with the retrospective design as its major limitation. However, over the study period all placentae from deliveries in our institute that fulfilled the inclusion criteria were included. Despite study population differences between the present and previous studies, several of our findings are in line with those reported before and will be discussed below.

The present study shows that the presence of concomitant PE or HELLP syndrome is associated with chronic inflammation of the foetal membranes. This phenomenon was originally described by Gersell and colleagues (141), but since then only one paper has reported on the occurrence of chronic lymphocytic inflammation of the foetal membranes in two IUGR placentae from pregnancies complicated by PE/HELLP syndrome (142). Early-onset PE has been associated with a pre-existent maternal pro-inflammatory phenotype and excessive production of pro-inflammatory cytokines and chemokines (reviewed by (143)). Recently, van Rijn and colleagues have reported that mutations of the maternal innate immune toll like receptor 4 (TLR4), which have been related to an inappropriate inflammatory pattern, are more common in women with a history of early-onset PE as compared to controls (144). Chronic inflammation of the foetal membranes may reflect activation of the maternal immune system in response to the semi-allogenic foetus or an infectious agent.

Infarction was found significantly less often in HELLP syndrome as compared to PE placentae which is in line with a recent report by Vinnars et al, even though that group included placentae with a gestational age range of 23-42 weeks (129). This finding is most likely due to earlier intervention due to worsening maternal clinical condition; management of PE is often more expectant than that of HELLP syndrome, providing more time for additional damage to occur in these already compromised placentae. This theory is supported by the fact that pregnancy was terminated for foetal reasons in 80% of PE as compared to 39% of HELLP syndrome pregnancies. A study by Smulian et al showed no difference between PE and HELLP placentae in the degree of infarction, but again, wide gestational age ranges were included (>50% delivered >32 weeks) and approximately 50% of women in their study developed HELLP syndrome *after* childbirth (128).

Apoptotic syncytial knotting is part of normal placental development and remodelling (145) and occurs when degenerating apoptotic nuclei accumulate and protrude from the villous surface (146). This phenomenon ensures that placental cells that are no longer functional are eliminated without causing a local inflammatory reaction in the mother (147). In our study, the presence of concomitant PE/HELLP syndrome was associated with increased knotting, indicating increased shedding of trophoblast. No difference in knotting was found between PE and HELLP syndrome placentae which is in line with data from Smulian et al (128). However, fascinatingly, HELLP syndrome placentae showed less apoptosis and more oxidative stress than PE placentae. Placental oxidative stress has been shown to be increased in both PE and HELLP syndrome (110;148) with highest levels in HELLP syndrome placentae (149). Furthermore, the degree of *maternal* oxidative stress has been shown to be associated with clinical disease severity (94;109) and very recently, *neonatal* oxidative stress has been shown to be increased in blood from infants born to mothers with HELLP syndrome as compared to PE (112). We therefore speculate that increased oxidative stress causes a switch from apoptotic to necrotic trophoblast shedding in HELLP syndrome placentae, thereby reducing the rate of apoptosis relative to the PE placentae (150). The extent of placental necrosis in these placentae is currently being studied.

Aponecrotic trophoblast causes an inflammatory response in the mother (147) which may provide an explanation for the more serious maternal condition of HELLP syndrome. Another aspect that most certainly plays a part in the more serious maternal condition of HELLP syndrome is the maternal innate immune system. Maternal TLR4 mutations have been shown to be 2.4 times more common in HELLP syndrome than in PE (144). Van Rijn and colleagues suggest that endogenous ligands (such as shed trophoblast) or exogenous (infectious) agents involved in the TLR4 pathway might be candidate

factors triggering HELLP syndrome. The role of TLR4 in the maternal response required to eliminate excess shedding of trophoblast remains to be elucidated (144).

The severity of IUGR (presence of ARED) is associated with increased presence of pathologic features characteristic of chronic hypoxia (increased numbers of NRBCs (151), reduced placental weight and distal villous hypoplasia). The NRBC results of the present study are in line with findings in neonatal blood (152;153) and show that severely elevated placental NRBCs are present significantly more often in ARED as compared to PED UA flow, indicating prolonged intrauterine stress due to placental insufficiency.
Severe IUGR has been shown to be associated with major defects in the development of gas-exchanging villi by stereology (154) and scanning electron microscopy (155). In the present study, distal villous hypoplasia was studied by light microscopy (as done previously by Todros et al (133) (villous pattern described in table 1)), and was found significantly more often in placentae with ARED UA flow which is in line with the stereological and electron microscopy findings (154;155). It is plausible that abnormal development of the villous tree causes increased placental resistance leading to severely abnormal UA flow.

Conclusion

Increasingly abnormal UA flow is associated with increased presence of pathologic features characteristic of chronic hypoxia (increased numbers of NRBCs, reduced placental weight and distal villous hypoplasia).
Both PE and HELLP syndrome are associated with chronic inflammation of foetal membranes which may reflect activation of the maternal immune system in response to the semi-allogenic foetus or an infectious agent. Increased knotting in both PE and HELLP syndrome placentae indicates increased shedding of trophoblast. HELLP syndrome was associated with more placental oxidative stress and less apoptosis as compared to PE. Increased oxidative stress may cause a switch from apoptotic to necrotic trophoblast shedding, thereby reducing the rate of apoptosis relative to PE. This is currently being studied.

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IS ANTENATAL STEROID TREATMENT EFFECTIVE IN PRETERM IUGR FOETUSES? A REVIEW OF THE LITERATURE

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Abstract

Background

There are no randomised studies on the effect of antenatal corticosteroids in preterm intrauterine growth restricted (IUGR) foetuses. Foetal lung maturation has been postulated to be enhanced in these foetuses, which may result in little benefit of steroid treatment. Furthermore, corticosteroid treatment may be detrimental, as has been shown in IUGR animal models.

Objective

To review the available literature on antenatal steroid treatment of the IUGR foetus

Methods

All available reports on antenatal steroid treatment of IUGR and small for gestational age (SGA) foetuses published prior to October 2007 were included in this review. IUGR foetuses are a subgroup of SGA foetuses that are small due to placental insufficiency which is reflected in abnormal Doppler examination of the umbilical artery.

Main outcome measures

Respiratory distress syndrome (RDS), intraventricular haemorrhage (IVH), necrotizing enterocolitis (NEC) and neonatal mortality.

Results

No difference in neonatal mortality was seen in any of the reviewed studies and RDS, IVH and NEC incidence did not differ between treated and untreated IUGR foetuses. In SGA foetuses, results on RDS incidence and intracranial outcome were inconclusive.

Conclusions

Antenatal steroid treatment does not seem to reduce neonatal mortality or morbidity in IUGR foetuses. In SGA foetuses, it remains unclear if antenatal steroid treatment is beneficial due to heterogeneous populations and treatment regimes. A randomised controlled trial should be performed to confirm prior results and answer further questions regarding antenatal steroid treatment of these foetuses.

Introduction

It has been postulated that intrauterine growth restriction (IUGR) may lead to enhanced foetal lung maturation (24). Chronic intrauterine stress may stimulate production of cortisol by the foetal adrenal gland with consequent lung maturation (98). In addition, IUGR foetuses are exposed to more maternal steroids through breakdown of the enzyme that normally prevents maternal cortisol from crossing the placenta (11-beta-hydroxysteroid dehydrogenase type II (11-βHSD II)) (156). Exogenous administration of glucocorticoids may therefore have no additional benefit in IUGR foetuses.

To date no clinical studies specifically studying IUGR foetuses have been able to confirm that clinical respiratory outcome is better (25-27;100). However, a recent study by our group shows that the lecithin/sphingomyelin (L/S) ratio is in fact higher in IUGR foetuses, indicating that biochemical measures of lung maturation are increased when placental insufficiency is present (157).

Administration of antenatal steroids to accelerate foetal lung maturation has been studied extensively and there is general consensus that steroids should be administered to women at risk of preterm birth (31-33). However, since the first trial by Liggins and Howie (34), IUGR foetuses have been excluded in all large randomised controlled trials. In IUGR animal models, antenatal steroids have been shown to reduce foetal brain growth, alter foetal (cerebral) blood flow and cause brain damage (35-37) . Furthermore, antenatal steroid exposure is hypothesised to be a key mechanism underlying the foetal origins of adult disease hypothesis, as is IUGR itself (158).

In human blood flow studies, preterm IUGR foetuses have been shown to exhibit divergent cardiovascular responses to antenatal steroid treatment. Wijnberger and colleagues found no effect of steroid treatment on foetal Doppler waveform patterns (159). Two other groups found that antenatal steroid administration altered resistance to fetoplacental blood flow (transient return of diastolic velocities in the umbilical artery in approximately 50-70% of foetuses (160;161)); however, it remains unclear if this alteration is detrimental (normalization of umbilical velocities may be at the cost of cerebral blood flow) or beneficial to the IUGR foetus (161).

If lung maturation is enhanced in IUGR foetuses and detrimental effects of antenatal steroid exposure can be expected in this population, should we then treat these foetuses with antenatal steroids? This question led us to review the available literature on antenatal steroid treatment of the IUGR foetus.

Methods

All human studies on antenatal steroid administration to mothers pregnant with IUGR foetuses prior to October 2007 were included in this review. Yet unpublished results from the study by van Stralen et al were also included (personal communication). A Pubmed search was performed using the following MeSH terms: "Foetal Growth Retardation", "Dexamethasone" and "Betamethasone". We decided to include both dexamethasone and betamethasone treatment in this analysis, because these steroids are both commonly used in clinical practice. Betamethasone, however, has been shown to cause a larger reduction in respiratory distress syndrome (RDS) incidence (33) and less neonatal cystic periventricular leukomalacia (PVL) (162) than dexamethasone.

Because literature on the IUGR foetus specifically is sparse, we also decided to include reports studying the small for gestational age (SGA) foetus. SGA has been defined as a foetal weight below the 10th percentile for gestational age (based on abdominal and head circumference or birth weight below 10th percentile for gestational age). IUGR foetuses are a subgroup of SGA foetuses with IUGR being defined as SGA due to placental insufficiency which is diagnosed by abnormal Doppler examination of the umbilical artery (163) or pathological examination of the placenta. It is important to realise that during the preterm period, SGA associated with normal umbilical blood flow is a different entity from that associated with abnormal flow, where those with abnormal flow have a higher risk of adverse outcome (164).

Outcome measures of interest included: RDS, intracranial outcome (intraventricular haemorrhage (IVH), intracranial haemorrhage (ICH), or PVL), necrotizing enterocolitis (NEC), neonatal death, survival without handicap at 2 years of age and physical growth and behaviour at school age.

If several studies reported on the same outcome variable a pooled odds ratio (OR) was calculated. This was done, both in pregnancies defined as SGA or IUGR, by combining outcome data of included studies and calculating a pooled OR and the 95% confidence interval (95% CI) of this pooled OR (Statistical Package for the Social Sciences SPSS version 14).

Table 1 Reported outcomes in the included articles

	Number of subjects		RDS			IVH/ICH/PVL			NEC			Neonatal death		
	No CCS (n)	CCS (n)	*No CCS*	*CCS*	*OR (95%CI)*	*No CCS*	*CCS*	*OR (95%CI)*	*No CCS*	*CCS*	*OR (95% CI)*	*No CCS*	*CCS*	*OR (95%CI)*
IUGR														
Schaap	62	62	40%	37%	*p*=0.8	15%	13%	*p*=1.0				24%	15%	*p*=0.2
Van Stralen	34	54	50%	42%	*1.2 (0.8-1.9)*	3%	8%	0.4 (0.1-3.4)	6%	6%	1.0 (0.2-5.8)	12%	9%	1.3 (0.4-4.4)
Torrance	28	112	39%	42%	0.5 (0.2-1.6)							7%	13%	1.4(0.3-6.9)
POOLED OR IUGR			**1.10 (0.70-1.71)**			**1.0 (0.41-2.42)**						**1.44 (0.78-2.68)**		
SGA *														
Ley	117	117			1.2 (0.6-2.3)			0.7 (0.3-1.8)						0.5 (0.2-1.3)
Spinillo	64	32	47%	31%	0.5 (0.2-1.3)	31%	7%	0.2 (0.04-0.8)						
Elimian	157	63	24%	27%	*p*=0.67	15%	22%	*p*=0.17	1.9%	1.6%	*p*=0.99	7%	8%	*p*=0.78
Torrance	19	146	42%	44%	0.6 (0.2-1.5)							13%	10%	0.7 (0.2-2.3)
POOLED OR SGA			**0.76 (0.52-1.11)**			**1.10 (0.58-2.07)**						**0.82 (0.44-1.67)**		

* The study by Bernstein et al was not included in this table, because number of subjects, percentages and ORs were not reported in the article

RDS: respiratory distress syndrome
OR: odds ratio
NEC: necrotizing enterocolitis
ICH: intracranial haemorrhage
SGA: small for gestational age
PVL: periventricular leukomalacia
IVH: intraventricular haemorrhage
IUGR: intrauterine growth restriction
CCS: antenatal steroid treatment

Results

Our search retrieved three and five articles on antenatal steroid treatment of IUGR and SGA foetuses, respectively. Except for Schaap et al (case-control study) (97) and Spinillo et al (prospective observational study) (96), all studies had a retrospective cohort design. In the study by Bernstein et al (84), no exact data on number of SGA foetuses and no data on ORs for the SGA subpopulation were mentioned in the paper. This study was therefore not included in the calculation of the pooled ORs for the SGA group. Further study design characteristics are described in the supplemental tables.

Respiratory distress syndrome

No difference in respiratory outcome was seen in any of the IUGR studies (97;100) (van Stralen et al (personal communication)) (pooled OR for RDS in IUGR studies was 1.10 (95% CI 0.70-1.71)); nor in the majority of reports on SGA foetuses (85;95;96;100) (the pooled OR for RDS in SGA studies was 0.76 (95% CI 0.52-1.11)) (table 1). Only one group found a significant reduction in RDS risk in steroid treated SGA foetuses. However, this steroid associated RDS risk reduction was smaller in SGA foetuses as compared to the steroid associated RDS risk reduction in appropriately grown infants (84).

Intracranial outcome

IVH incidence did not differ between treated and untreated IUGR foetuses (97) (van Stralen et al; pooled OR 1.0 (95% CI 0.41-2.42)). For SGA foetuses, intracranial outcome was better in steroid treated foetuses in two reports (84;96), but unchanged in two others (85;95) (table 1). Pooling of these four results, gives an OR for IVH in SGA foetuses of 1.10 (95% CI 0.58-2.07).

Necrotizing enterocolitis

NEC incidence did not differ between treated and untreated foetuses in IUGR (van Stralen et al) or SGA foetuses (84;95) (table 1). For both IUGR and SGA foetuses, only one study provided the number of infants with NEC, which made pooling of results impossible for both groups.

Neonatal death

No significant difference in neonatal death was found between groups in any of the IUGR (97;100) (van Stralen et al) (pooled OR 1.44 (95% CI 0.78-2.68)) or SGA studies (85;95;96;100) (pooled OR 0.82 (95% CI 0.44-1.67)) (table 1).

Survival without handicap at two years and outcome at school age

Merely one article reported on long term outcome after steroid treatment (97). Survival without handicap at two years was more frequent in IUGR infants treated with antenatal steroids (82% in the treated group versus 65% in the untreated group (OR 3.2 (95% CI 1.1-11.2)). At school age there was no difference in behaviour between groups. However, physical growth beneath the tenth percentile was significantly more frequent after steroid treatment (OR 5.1 (95% CI 1.4, 23.8)) (97).

Discussion

Literature on antenatal steroid treatment of the IUGR foetus is sparse. No randomised trials have been performed in this specific population, which is possibly due to early reports of adverse foetal effects of steroids in women with severe hypertension and growth restricted foetuses (34).

From the available studies, we can conclude that antenatal steroids do not seem to reduce neonatal morbidity or mortality (97;100) (van Stralen et al (personal communication)) in the IUGR foetus. At two years of age, a reduction in handicapped children has been reported in steroid treated IUGR foetuses; however, information on the longer term is not available because follow up of this cohort at school age did not include assessment of handicaps. At this age, no differences were seen in behaviour, however, physical growth below the 10th percentile in steroid treated infants was significantly more frequent (97). Long term follow-up into adulthood has not been reported which is of importance because antenatal treatment is hypothesised to be a key mechanism underlying the foetal origins of adult disease hypothesis (158). Long term follow up of appropriately grown foetuses, however, failed to show any detrimental effects of a single course of antenatal steroids (165;166). Repeat courses of antenatal steroids may have detrimental effects (167;168), but benefits and risks need to be determined in future studies (33).

Antenatal steroid treatment does not seem to reduce RDS incidence in IUGR foetuses, possibly because lung maturity is already enhanced in these foetuses due to raised endogenous corticoid production associated with chronic intrauterine stress and breakdown of 11-βHSD II (24;156). The incidence of IVH was also similar between treated and untreated IUGR foetuses (97) (van Stralen et al). It is possible that IUGR itself not only enhances foetal lung maturation but also matures the central nervous system (169). This enhanced maturation may stabilise the endothelium of the germinal matrix causing the matrix to become less vulnerable to cerebral blood pressure fluctuations which are related to the occurrence of intracranial haemorrhage.

IUGR can be accompanied by maternal hypertensive disease of pregnancy. A recent report showed that IUGR infants born to mothers with HELLP syndrome (haemolysis, elevated liver enzyme, low platelets) have a significantly worse respiratory outcome (100) as compared to IUGR infants born to mothers with normotension or pre-eclampsia, which may be due to increased oxidative stress (112). Amorim and colleagues have shown that antenatal bethamethasone is a safe and efficient treatment in patients with severe pre-eclampsia between 26 and 34 weeks' gestation if immediate delivery is not indicated (170). The latest Cochrane review confirms these findings (33); to our knowledge a randomised trial in women with HELLP syndrome has not been performed.

No difference in neonatal mortality was found in any of the SGA studies. Results on neonatal morbidity in this population are inconclusive possibly resulting from the heterogeneous population in the studies. The SGA studies do not report whether cases were evaluated for signs of placental insufficiency (as shown by abnormal umbilical blood flow Doppler studies and/or placental pathology), most probably resulting in a heterogeneous SGA/IUGR population. Furthermore, in two reports, type of steroid treatment was not strictly defined and assignment to the steroid treated group merely required that antenatal steroid treatment had been initiated and not necessarily completed (supplemental data (84;85)). This may have resulted in treatment heterogeneity in the steroid group. Importantly, the study by Bernstein et al was the only study that found a significant reduction in RDS and IVH risk in steroid treated SGA foetuses (84). However, the steroid associated RDS risk reduction was smaller in SGA foetuses as compared to the steroid associated RDS risk reduction in appropriately grown infants. Unfortunately, these observations could not be used in calculation of the pooled ORs, because no exact data on number of SGA foetuses and no data on ORs for the SGA subpopulation were provided in the paper.

Additional evidence on the effectiveness of steroid treatment according to birth weight percentile for gestational age has been published by Schutte et al. This article reports that betamethasone treatment reduced RDS-incidence in infants with birth weight > 25th percentile only (171). Hence, it may be worthwhile if data from the large randomised trials are reanalyzed according to birth weight percentile.

On the basis of this literature review, we propose that a randomised controlled trial should be performed to study the effect of antenatal steroid treatment in IUGR and SGA foetuses. The inclusion criteria of this trial should focus on the distinction between these two populations and should evaluate both short and long term outcome.

In conclusion, so far, there is insufficient evidence that antenatal steroid administration reduces neonatal morbidity and mortality in IUGR foetuses. Animal studies even suggest a detrimental effect of steroid administration on foetal brain development in

IUGR foetuses (35-37). In addition, it remains unclear if antenatal steroid treatment is beneficial in SGA foetuses which may be due to heterogeneous study populations and treatment regimes. A randomised controlled trial should be performed to investigate whether treatment is beneficial in IUGR foetuses and to answer further questions regarding antenatal steroid treatment of SGA foetuses.

Supplemental tables

Study	Schaap 2001
Methods	Type of study: case-control Sample size: 120 infants Losses to follow-up: 9 losses at school age follow-up
Participants	Location: two tertiary care centres in the Netherlands Time frame: 1984-1999 Eligibility criteria: live-born singletons with growth restriction due to placental insufficiency (confirmed by pathological examination of the placenta), who were delivered by caesarean section because of foetal distress before the beginning of labour at a gestational age of 26 through 31 weeks Exclusion criteria: admittance of the mother less than 24 hours before delivery, foetal death, foetal distress at admission, abruptio placentae, lethal congenital anomalies, congenital infections. Total number of subjects: 62 cases and 62 matched controls (matched for birth weight, sex and year of birth)
Interventions	Betamethasone treatment depended on the decision of the attending obstetrician and consisted of two doses of 12.5 mg IM injection with a 24-hour interval. The treated group consisted of all infants whose mothers had been given betamethasone more than 24 hours up to 7 days before birth. The control group did not receive antenatal betamethasone treatment .
Outcomes	RDS, bronchopulmonary dysplasia, intracerebral haemorrhage grades 3 and 4, sepsis, survival at discharge, two year follow up and long term follow-up at school age.

Study	van Stralen (personal communication)
Methods	Type of study: retrospective cohort
Participants	Location: tertiary care centre in the Netherlands Time frame: 2001-2005 Eligibility criteria: singletons with GA < 34 weeks and birth weight <1500 grams and abnormal Doppler ultrasound examination of the umbilical artery Growth restriction was defined as a U/C ratio > 0.725. The U/C ratio was calculated by dividing the PI of the UA by the PI of the MCA. Exclusion criteria: multiple pregnancies, foetuses with major congenital anomalies or infection and deliveries with insufficient data. Total number of subjects: 54 steroid treated, 34 non-treated
Interventions	Betamethasone treatment depended on the decision of the attending obstetrician and consisted of two doses of 12.5 mg IM injection with a 24-hour interval. The treated group consisted of all infants whose mothers had been given betamethasone more than 24 hours up to 7 days before birth. The control group did not receive antenatal betamethasone treatment.
Outcomes	RDS, neonatal death, adverse neonatal outcome (defined as minimal one of the following: moderate or severe bronchopulmonary dysplasia, NEC grade 2 or higher or retinopathy of prematurity grade 3 or higher).

Study	Torrance 2007
Methods	Type of study: retrospective cohort
Participants	Location: tertiary care centre in the Netherlands Time frame: 1999-2003 Eligibility criteria: Infants with gestational age < 34 weeks and birth weight <10th percentile for gestational age with or without abnormal Doppler examination of the umbilical artery. Exclusion criteria: congenital, chromosomal or syndromal abnormalities Total number of subjects: 142 IUGR foetuses
Interventions	Betamethasone treatment depended on the decision of the attending obstetrician and consisted of two doses of 12 mg IM injection with a 24-hour interval. The treated group consisted of all infants whose mothers had been given betamethasone more than 24 hours before birth. The control group did not receive antenatal betamethasone treatment.
Outcomes	RDS, surfactant treatment, ventilation, chronic lung disease and neonatal death

Study	Elimian 1999
Methods	Type of study: retrospective cohort
Participants	Location: New York Medical College, New York Time frame: 1990-1997 Eligibility criteria: all women who delivered infants weighing 1750 grams or less at birth SGA was defined as birth weight <10th percentile for gestational age Exclusion criteria: not specified Total number of subjects: 220 SGA foetuses
Interventions	Antenatal steroid treatment depended on the decision of the obstetrician and consisted of 2 12 mg intramuscular doses of betamethasone 24 hours apart with a repeat course 7 days from the first dose if undelivered. The treated group consisted of all infants whose mothers had been given betamethasone more than 24 hours before birth (n=63). The control group did not receive antenatal betamethasone treatment (n=157).
Outcomes	RDS, IVH/PVL, major brain lesion, NEC, neonatal sepsis, neonatal death

Study	Spinillo 1995
Methods	Type of study: prospective observational
Participants	Location: University of Pavia, Italy Time frame: 1988-1993 Eligibility criteria: preterm delivery between 24-34 weeks, liveborn, non-malformed infants, either optimal steroid treatment or no steroids treatment, planned delivery for patients with medical complications of pregnancy (e.g. FGR, PE). FGR was defined as an abdominal circumference or cephalic circumference <10th percentile for GA and birth weight<10th percentile Exclusion criteria: triplets, pregnancies complicated by severe abruption or eclampsia Total number of subjects: 32 steroid treated, 64 non-treated
Interventions	Optimal steroid treatment consisted of 2 intramuscular doses of 12 mg betamethasone or dexamethasone with the first dose >48 hours and <7 days before delivery. Steroids were administered at the discretion of the attending physician.
Outcomes	RDS, IVH, neonatal death

Study	Bernstein 2000
Methods	Type of study: retrospective cohort
Participants	Location: Vermont Oxford Database, Canada Time frame: 1991-1996 Eligibility criteria: Singletons without major anomalies born between 25 and 30 weeks' gestation with birth weight < 10th percentile for gestational age Exclusion criteria: not specified Total number of subjects: not specified
Interventions	Documentation on glucocorticoid administration required only that a prenatal course of corticosteroids be initiated before delivery; it did not require completion of this course.
Outcomes	RDS, IVH, NEC and death.

Study	Ley 1997
Methods	Type of study: retrospective cohort
Participants	Location: University hospital Lund, Sweden Time frame: 1985-1994 Eligibility criteria: live-born SGA infants with gestational age < 33 weeks SGA: not defined Exclusion criteria: not specified Total number of subjects: 117 steroid treated, 117 non-treated
Interventions	Antenatal steroid treatment definitions not clearly specified. If one or more doses of steroids were administered the infant was assigned to the treated group.
Outcomes	Mortality, RDS, IVH grade 3 or PVL

7

LACTATE TO CREATININE RATIO IN AMNIOTIC FLUID: A PILOT STUDY

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Abstract

Background

Measurement of amniotic fluid lactate concentration in complicated pregnancies may provide information on the extent of foetal acidemia. However, normalisation for amniotic fluid volume may be necessary by calculating the lactate:creatinine (L:C) ratio.

Methods

Amniotic fluid lactate and creatinine concentrations were obtained at caesarean section and were compared to lactate concentration in simultaneously collected arterial cord blood of 28 term and 10 preterm pregnancies.

Results

Cord blood lactate was not correlated to amniotic fluid lactate, but was correlated to the L:C ratio in the complete study population (R=0.54, p=0.001) and the subgroups. Correlation was strongest in a preterm intrauterine growth restricted subgroup (n=7, R=0.83, p=0.02).

Conclusion

The L:C ratio is more accurate in estimating foetal lacticemia than the amniotic fluid lactate concentration. When transabdominal amniocentesis is performed for determination of foetal lung maturity, the L:C ratio can be determined simultaneously in merely 2 ml of amniotic fluid. In future, simultaneous non-invasive assessment of foetal acidosis and foetal lung maturity may become possible by measurement of the L:C and L/S ratios via magnetic resonance spectroscopy and/or infrared spectroscopy.

Introduction

Foetal acidemia may develop in intrauterine growth restricted (IUGR) foetuses as a result of diminished transport of nutrients and oxygen to the foetus due to placental insufficiency. With increasing placental insufficiency, a point may be reached where the disadvantages of a detrimental intrauterine environment outweigh the disadvantages of the delivery of an immature infant. In each individual case, clinicians must weigh the advantages and disadvantages of prolonged intrauterine life and come to a decision on the optimal time of delivery for the foetus (10).

Determining foetal lung maturity can aid in this decision. This can be assessed in amniotic fluid obtained via transabdominal amniocentesis. An earlier study by our group, showed that the lecithin to sphingomyelin (L/S) ratio is >2 (indicating foetal lung maturity) in half of the IUGR foetuses between 30 and 32 weeks of gestation (172). Simultaneous measurement of amniotic fluid lactate concentration may provide insight into the extent of foetal lacticemia. However, this concentration may be influenced by the amount of amniotic fluid volume which is often diminished in IUGR. One approach to ensure that differences in amniotic fluid metabolite levels are not due to a different rate of foetal urine production is to calculate a ratio of the amniotic fluid metabolite of interest to creatinine (173). The lactate to creatinine (L:C) ratio may therefore be more accurate for determination of the foetal condition in complicated pregnancies. This ratio has not been studied before as an indicator of foetal wellbeing in IUGR. However, in postnatal life the urinary L:C ratio has been shown to be predictive for the development of hypoxic-ischemic encephalopathy in asphyxiated newborns (56).

The aim of the present study was to measure the L:C ratio and lactate concentration in amniotic fluid obtained during caesarean section and to correlate these parameters with foetal lactate concentrations measured simultaneously in arterial cord blood.

Methods

In this pilot study, amniotic fluid samples were collected prospectively from pregnant women undergoing caesarean section. After obtaining informed consent, women with uncomplicated pregnancies undergoing elective caesarean section at term (for instance for breech presentation or repeat caesarean section) and women undergoing caesarean section prematurely with an estimated foetal weight <p10 were included in the study. Foetuses with chromosomal and syndromal abnormalities were excluded.

Amniotic fluid samples (2 ml) were collected during caesarean section. Samples that

were contaminated with blood or meconium were discarded. Arterial umbilical cord blood samples were collected simultaneously. In all materials, creatinine and lactate concentrations were measured immediately (SI units).
Metabolite concentrations were analyzed in two gestational age (GA) groups: term foetuses (GA>37 weeks) (1) and preterm foetuses (GA≤34 weeks) (2). The latter group was divided into foetuses with intrauterine growth restriction (IUGR) associated with placental insufficiency (if the pulsatility index (PI) of the umbilical artery was repeatedly more than 2 standard deviations above the median) and foetuses that were small for gestational age (SGA) and demonstrated normal umbilical artery PI.
Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS version 14.0). Pearson's correlations were determined or non-parametric Spearman's correlations where appropriate. Statistical significance was accepted when $p<0.05$.
The study was approved by the Medical Ethics Committee of the University Medical Centre Utrecht, the Netherlands.

Results

After obtaining informed consent, 54 pregnant women were included in the study and 56 amniotic fluid samples were collected (two twin pregnancies). In 18 cases, the arterial cord blood lactate could not be measured (because too little arterial cord blood was available or due to errors in blood sampling). These cases were therefore excluded from analysis (including both twin pregnancies).
All 28 infants delivered electively at term were healthy. Median GA at birth in this group was 274 days (range 272-278) and median birth weight 3480 grams (range 2855-4630). Term infants from mothers with insulin treatment for diabetes were admitted to the neonatal medium care unit for monitoring of blood glucose levels (n=3). Group 2 consisted of 10 infants with a median GA of 215 days (range 199-231) and median birth weight of 1100 grams (range 760-1380). In this group, seven infants were IUGR, five infants were delivered for nonreassuring foetal monitoring and nine infants were delivered for maternal reasons (pre-eclampsia or HELLP syndrome). All IUGR infants were admitted to the neonatal intensive care unit.
The L:C ratio decreased with increasing gestational age (correlation coefficient: -0.72, *p* value 0.000). No correlation was found between arterial cord blood lactate and amniotic fluid lactate concentration in the complete study population (R=0.25, p value 0.13), group 1 (R=0.19, p value 0.34) or group 2 (R=0.46, p value 0.18). In contrast,

arterial cord blood lactate concentration was significantly correlated to the L:C ratio in the complete study population (R=0.54, *p* value 0.001), group 1 (R=0.59, *p* value 0.001) and group 2 (R=0.72, *p* value 0.02) (see figure). Correlation was strongest in the IUGR subgroup (R=0.83, *p* value 0.02).

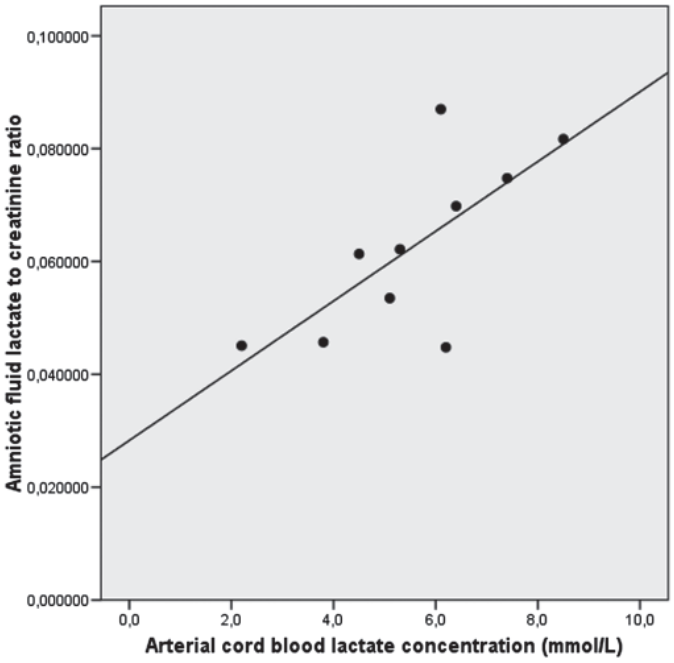

Figure 1 Correlation between arterial cord blood lactate concentration and amniotic fluid lactate:creatinine ratio of preterm foetuses

Discussion

The present study indicates that the amniotic fluid L:C ratio decreases with increasing gestational age and that this ratio is significantly correlated with arterial umbilical cord lacticemia. In contrast, the amniotic fluid lactate concentration was not correlated with foetal lacticemia.

This finding may be explained by the fact that the amniotic fluid lactate concentration is influenced by the amniotic fluid volume, similar to the lecithin concentration (174;175). Normalization for amniotic fluid volume was performed by calculating the amniotic fluid lactate to creatinine ratio (173).

A good deal of research into normal biochemical composition of amniotic fluid was performed many years ago and it has shown that metabolite concentrations change during normal pregnancy. With increasing gestational age, lactate concentration falls whereas creatinine has been shown to increase (176). This means that the L:C ratio can be expected to fall with increasing gestational age, as was shown in the present study. To our knowledge, this study is the first to report data on the correlation between simultaneously measured arterial umbilical cord lactate concentrations and amniotic fluid lactate and creatinine concentrations after caesarean section delivery of both term and SGA preterm infants. The present study shows that the L:C ratio is of significance in both populations. Unfortunately, this pilot study was not designed to correlate foetal lacticemia results with neonatal outcome, which obviously is of great interest. The urinary L:C ratio, however, has been shown to be predictive for the development of hypoxic-ischemic encephalopathy in asphyxiated newborns (56). Future studies should focus on measuring the amniotic fluid L:C ratio and on correlating this ratio with neonatal outcome.

From a practical point of view, the L:C ratio can be determined in merely 2 ml of amniotic fluid when transabdominal amniocentesis is performed for determination of foetal lung maturity. In future, the L:C ratio may become important, as recent studies have demonstrated the capability to measure various amniotic fluid metabolites (including lecithin, lactate and creatinine) non-invasively via magnetic resonance spectroscopy (MRS) (53-55) or infra red spectroscopy (177). These non-invasive diagnostic tools could become valuable alternatives to invasive amniocentesis for the simultaneous assessment of foetal asphyxia and foetal lung maturity.

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8

PREDICTORS FOR OUTCOME AT TWO YEARS OF AGE AFTER EARLY-ONSET INTRAUTERINE GROWTH RESTRICTION

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Abstract

Objective

To examine the relative importance of antenatal and perinatal variables on short and long term outcome of preterm growth restricted foetuses with umbilical artery Doppler abnormalities.

Methods

Cohort study of 180 neonates with birth weight <10th percentile, gestational age (GA) <34 weeks and abnormal Doppler ultrasound examination of the umbilical artery (UA). Various antenatal and perinatal variables were studied in relation to short and long term outcome.

Results

Neonatal mortality was significantly associated with GA at delivery, absent/reversed UA Doppler flow and abnormal ductus venosus blood flow velocities. Both infant and overall mortality were significantly associated with birth weight, abnormal ductus venosus examination and placental villitis. Placental villitis was the only parameter that was significantly associated with necrotizing enterocolitis. GA, birth weight, male sex and absent/reverse UA Doppler flow showed significant associations with respiratory distress syndrome. Abnormal neurodevelopmental outcome at 2 years of age was only associated with birth weight, UA pH<7.00 and placental villitis.

Conclusion

The present study confirms that GA and Doppler findings are important predictors for short term outcome in growth restricted foetuses. In addition, presence of placental villitis may aid neonatologists in early identification of infants at increased risk of necrotizing enterocolitis, death and abnormal neurodevelopment at 2 years of age. Abnormal neurodevelopment was related to weight and acidosis at birth indicating that the severity of malnutrition and foetal acidosis affect long term outcome.

Introduction

Timing of delivery of the intrauterine growth restricted (IUGR) infant is still a matter of debate, especially during the very preterm period. Benefits of further intrauterine maturation have to be weighed against complications that may occur due to prolonged malnutrition and hypoxia. Standardised management protocols are not available, so individualisation based upon known predictive factors is necessary to optimise the timing of delivery in each individual case.

Data from the literature indicate that gestational age (GA) at delivery, birth weight, abnormal Doppler waveform patterns and/or abnormal foetal heart rate (FHR) patterns are strong predictors for neonatal outcome (10;26;38-48).

Recently, Baschat et al studied a large group of growth restricted foetuses with placental insufficiency and confirmed that GA, birth weight and ductus venosus Doppler indices remain the most important parameters in predicting neonatal outcome (39). Although this study was large, it was a multicentre study, with a focus on foetal cardiovascular parameters and merely studied outcome until discharge from the hospital. The authors mention in their discussion that additional predictors may need to be considered, because the combination of foetal cardiovascular and known neonatal factors accounted for only 40% of adverse neonatal outcomes in their study.

The present study was performed to examine the relative importance of foetal and perinatal variables, including placental features, on outcome in a cohort of liveborn IUGR infants with early-onset placental insufficiency delivered before 34 weeks of gestation. Outcome was not only evaluated in the neonatal period but also included neurodevelopment at 2 years of age.

Methods

The cohort consisted of all liveborn neonates born between 1st January 1997 and 31st December 2004 with birth weight <10th percentile for GA (88), GA< 34 weeks and umbilical artery (UA) Doppler >2 SD from the reference ranges (90), admitted to the neonatal intensive care unit (NICU) of the University Medical Centre, Utrecht, the Netherlands. Cases with maternal infection, preterm premature rupture of membranes, chromosomal abnormalities, foetal deaths, twins/triplets and infants with major congenital anomalies were excluded.

The following perinatal factors were recorded from the patient notes: GA at delivery (established by menstrual history and/or from the results of first trimester

ultrasonographic examination), parity, route of delivery, antenatal steroid treatment, maternal hypertensive disease, Doppler indices, last cardiotocographic (CTG) tracing (within 24 hours) before delivery, birth weight to placental weight ratio according to reference values (178), degree of placental infarction and the occurrence and grade of chronic placental villitis of unknown etiology (placental VUE).

Antenatal steroid treatment was considered to be complete if the mother had received two injections of 12mg betamethasone 24 hours apart and more than 24 hours before delivery. Maternal hypertensive disease was defined as pregnancy induced hypertension (PIH), pre-eclampsia (PE) or HELLP-syndrome (haemolysis, elevated liver enzymes, low platelets) (89). Doppler indices were determined from the last measurement of the pulsatility index (PI) of UA, middle cerebral artery (MCA) and ductus venosus (DV) in the week before birth. Elevation of the UA PI >2 SD above the GA mean was considered to be abnormal and UA end diastolic flow was classified as being present or absent/reverse. For the MCA, PI >2 SD below the GA mean was classified as 'brainsparing' and a PI of the DV >2 SD above the GA mean was considered to be abnormal. The CTG tracing before delivery was classified as: 1. normal; 2. reduced variability or decelerative; and 3. strongly reduced variability with decelerations ((pre)terminal) (179;180). All placentae were examined by a perinatal pathologist (PN) who was blinded to clinical details except for gestational age at delivery. Percentage of placental infarction is determined routinely in all placentae from complicated pregnancies. Presence and extent of VUE were assessed according to a specific research protocol on a scale of 0-3 (absent, minimal, moderate, severe) (slightly modified from Knox et al (181)).

Neonatal and postneonatal outcome data recorded included: birth weight, sex, Apgar scores at 1 and 5 minutes, umbilical cord arterial pH, occurrence and grade of respiratory distress syndrome (RDS) (according to the classification of Giedion et al (91)), occurrence and grade of necrotizing enterocolitis (NEC) (according to the criteria of Bell (182)), occurrence and grade of intraventricular haemorrhage (IVH) (according to the classification of de Vries et al (183)), occurrence and grade of periventricular leukomalacia (PVL) (according to the classification of de Vries et al (184)), occurrence and grade of retinopathy of prematurity (ROP) (according to the International Classification of Retinopathy of Prematurity (185)), neonatal death (within the first 28 days of life), infant death (between 28 days and 1 year of life) and overall death (up to 2 years after birth). Severe neonatal complication was defined as the presence of one of the following: RDS ≥grade 3, IVH ≥grade 3, NEC ≥grade 2 or ROP ≥grade 3 ('severe composite morbidity').

The following neurodevelopmental data were recorded: cranial ultrasound (evaluation by a neonatologist blinded to all other study variables) and neurological exam at term

age (corrected for gestational age at birth), evaluation of development according to the Griffiths Mental Development Scale (186) at 18, 24 and 36 months and/or the Bayley Scales of Infant Development-II-NL (BSID-II-NL) (187) at 2 years of age. If children were tested more than once, the score at 2 years of age was used. If this score was not available, the DQ at 3 years or - if also not available – at 18 months was taken. The Griffiths Scale was used to assess cognitive development of children with GA ≥30 weeks and birth weight ≥1000g. The mental scale of the BSID-II-NL was used to assess cognitive development of children with GA <30 weeks and/or birth weight <1000g. Normal development was defined as a mental developmental index (MDI) (187) or developmental quotient (DQ) (186) > 85.

In case of incidental missing data, values were estimated with multiple regression analysis, using other available patient characteristics. This procedure to handle missing data is called imputation and it is a commonly used adequate technique (188).

Statistical analysis was performed in the Statistical Package for the Social Sciences (SPSS) version 14.0. For each outcome of interest, a maximum of 8 known predictors were selected from the literature and a maximum of 5 additional variables of pathophysiological notion were added. These variables were analysed in univariate logistic regression or Chi square analysis. Subsequently, all variables with a univariate *p* value <0.25 were included in a multivariate logistic regression analysis. Variables with a *p* value <0.05 and odds ratios (OR) with 95% confidence interval (CI) not inclusive of unity in the multivariate analysis were considered to be statistically significant. For continuous variables identified as significant contributors, receiver operating characteristics (ROC) curves were computed from which the area under the curve (AUC) and predictive cut-offs were determined (189).

Results

The study group consisted of a tertiary referral cohort of 180 foetuses. Antenatal and perinatal characteristics of the foetuses are shown in table 1a. All infants were born by caesarean section for maternal and/or foetal reasons. Postnatal outcome data were categorised into the following groups: 1) neonatal and infant mortality; 2) neonatal morbidity; and 3) neurodevelopmental outcome (table 1b).

Table 1a Antenatal and perinatal characteristics

Number of patients (n)	180
Male sex	100 (56%)
Birth weight (M; SD)	875±217
Birth weight <p2.3	70 (39%)
GA at delivery in weeks (M; SD)	30 2/7± 13 days
Complete antenatal steroid treatment	137 (76%)
Nulliparity	135 (75%)
Umbilical artery absent/reverse flow	76 (42%)
Brainsparing	151 (84%)
Ductus venosus > 2 SD	73 (41%)
Oligo- or anhydramnios	133 (74%)
Abnormal CTG	123 (68%)
Maternal hypertensive disease:	
PIH	16 (9%)
PE	57 (32%)
HELLP	64 (36%)
Ratio birth / placental weight (M; SD)	4.7±1.2
Placental infarction > 5%	80 (44%)
Placental VUE	40 (22%)
Grade I	34 (19%)
Grade II	5 (3%)
Grade III	1 (0.5%)
Apgar <5 at 1'	29 (16%)
Apgar <6 at 5'	5 (3%)
Umbilical cord arterial pH <7.00	10 (6%)

GA gestational age; CTG cardiotocogrpahy; PIH pregnancy induced hypertension; PE pre-eclampsia; HELLP haemolysis elevated liver enzymes low platelets; VUE villitis of unknown aetiology

Mortality

With increasing GA neonatal mortality decreased whereas infant mortality was fairly constant across gestation. Overall mortality decreased from 67% at 26 weeks to 0% from 32 weeks onwards (figure 1). Multivariate logistic regression revealed that neonatal mortality was significantly associated with GA at delivery, absent/reversed UA and abnormal DV flow. By ROC curve analysis, gestational age greater than 29 0/7

weeks provided the best prediction of neonatal survival (sensitivity 74.4%, specificity 65.0%, AUC 0.78, 95% CI [0.68-0.88], P<0.001). Both infant and overall mortality were significantly associated with birth weight, abnormal DV flow and placental VUE. Finally, overall mortality was also associated with severe composite morbidity (table 2). By ROC curve analysis, birth weight greater than 800 grams provided the best prediction of overall survival (sensitivity 64.1%, specificity 66.6%, AUC 0.73, 95% CI [0.64-0.82], P<0.001). The four variables that were associated with overall death accounted for 40% of overall deaths in this study.

Table 1b Outcome

Mortality	
Neonatal death	17 (9%)
Infant death	7 (4%)
Morbidity	
Severe composite morbidity	50 (28%)
IVH	28 (16%)
IVH grade ≥ 3	0
PVL	66 (37%)
PVL grade ≥ 2	2 (1%)
ROP	30 (17%)
ROP grade ≥ 3	2 (1%)
NEC	25 (14%)
NEC grade ≥ 2	12 (7%)
RDS	74 (41%)
RDS grade ≥ 3	37 (21%)
NICU days (M; SD)	21.7 ± 15.8
Neurodevelopment	
Abnormal cranial ultrasound †	26/127 *
Abnormal neurological exam at term	60 (38%)
Number of surviving infants at 2 years	156
Abnormal folllow-up at 2 years:	37 (24%)
Cerebral palsy	1 (0.6%)

IVH intraventricular haemorrhage; PVL periventricular leukomalacia; ROP retinopathy of prematurity; NEC necrotizing enterocolitis; RDS respiratory distress syndrome; NICU neonatal intensive care unit
† Abnormal or suspect cranial ultrasound at term or at 3 months of age (in the outpatients follow up clinic): data is incomplete because some children had been transferred to other hospitals before they reached term age.
* For parameters with missing cases the total amount of cases is given

Neonatal morbidity

Severe composite morbidity was present in 28% of cases. As gestational age advanced, a fall in severe complications was observed from 67% at 26 weeks to 0% from 31 2/7 weeks onwards. Multivariate logistic regression revealed significant associations

between severe complications and GA, male sex and abnormal cardiotocography. Together these variables accounted for 43% of severe neonatal outcomes in this study population.

Associations between antenatal risk factors and individual morbidity items can be found in table 3. IVH occurred in 28 infants (16%); however none had IVH grade ≥3. ROP was diagnosed in 30 infants (17%) of whom 2 had ROP grade ≥3 (1%). GA was the only parameter that was significantly associated with both IVH and ROP. NEC occurred in 25 infants (14%) of whom 12 infants developed NEC ≥ grade 2 (7%) and 6 (3.5%) required surgery. Placental VUE was significantly associated with NEC. In total, 7 infants with NEC died; 5 of these infants had signs of VUE in the placenta. Seventy-four infants developed RDS (41%) of whom 37 developed RDS grade 3 or higher. GA, birth weight, male sex and absent/reverse UA Doppler flow showed a significant association with RDS. Antenatal steroid treatment, administered to 76% of the population, was not significantly associated with RDS.

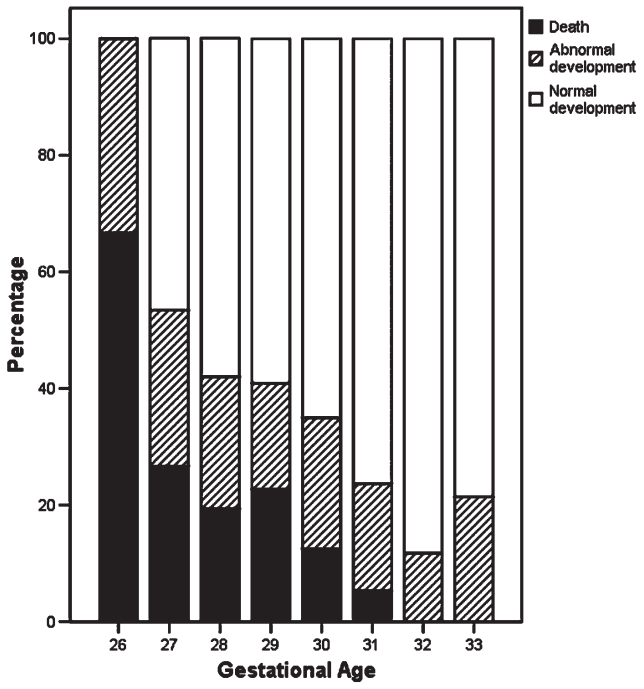

Figure 1 Death, abnormal and normal development per gestational age week.

Table 2 Predictors for mortality

	Neonatal death	Infant death	Overall death
Predictors		**OR (95% CI)**	
GA	0.5 (0.3-0.7)	NS	NS
BW	NS	0.4 (0.2-0.8)	0.5 (0.4-0.7)
UA absent/reverse	3.5 (1.1-12)	NS	NS
DV abnormal	9.7 (1.9-49)	19.2 (1.6-226)	13.0 (3.1-54)
Severe composite morbidity	NS	NS	2.8 (1.02-7.9)
Birth weight to placental weight ratio	NS	NS	NS
Placental VUE	NS	11.2 (1.7-72)	4.0 (1.3-13)
Placental Infarction >5%	NS	NS	NS
NEC	NS	NS	NS
Abnormal CTG	NS	NS	NS
Apgar 5'<6	NS	NS	NS
UC pH <7.00	NS	NS	NS
Male sex	NS	NS	NS

GA: gestational age
BW: birth weight
UA: umbilical artery absent/reverse end diastolic flow
DV abnormal: ductus venosus pulsatility index >2 standard deviations above the GA mean
Placental VUE: placental villitis of unknown aetiology
Placental infarction >5%: infarction of more than 5% of the placenta
NEC: occurrence and grade of necrotizing enterocolitis (according to the criteria of Bell)
CTG: cardiotocography
UC pH: pH of the umbilical artery
NS: non significant

Neurodevelopmental outcome

Development at 2 years of age was normal in 76% of the surviving infants. Intact survival increased from 0% at a GA of 26 weeks to 80% at 33 weeks (see figure). At 2 years of age, one child had developed cerebral palsy.

Abnormal neurodevelopment at 2 years of age was associated with birth weight, UA pH<7.00 and placental VUE (table 4). These three variables accounted for 22% of abnormal development at 2 years of age. By ROC curve analysis, birth weight greater than 835 grams provided the best prediction of overall intact survival at 2 years (sensitivity 65.5%, specificity 64.9%, AUC 0.70, 95% CI [0.61-0.79], P<0.001). IVH, PVL, NEC and neurological examination at term were not associated with neurodevelopmental outcome at 2 years of age.

Table 3 Predictors for morbidity

Predictors	Severe composite morbidity	IVH	ROP	NEC	RDS
			OR (95% CI)		
GA	0.4 (0.3-0.5)	0.7 (0.5-0.9)	0.6 (0.4-0.9)	NS	0.3 (0.2-0.4)
BW	NS	NS	NS	NS	1.5 (1.06-2.0)
UA absent/reverse	NS	NS	NS	NS	2.4 (1.1-5.5)
DV abnormal	NS	NS	NS	NS	NS
Apgar 5'<6	NS	NS	NS	NS	NS
UC pH <7.00	NS	NS	NS	NS	NS
Male sex	3.4 (1.4-8.0)	NS	NS	NS	2.3 (1.02-5.3)
Abnormal CTG	3.6 (1.4-9.3)	NS	NS	NS	NS
HELLP	NS	NS	NS	NS	NS
CCS	NS	NS	NS	NS	NS
VUE	NS	NS	NS	2.6 (1.0-6.7)	NS

GA: gestational age
BW: birth weight
UA absent/reverse: umbilical artery absent/reverse end diastolic flow
DV abnormal: pulsatility index of the ductus venosus >2 standard deviations above the GA mean
UC pH: pH of the umbilical artery
CTG: cardiotocography
HELLP: haemolysis, elevated liver enzymes, low platelets syndrome
CCS: complete antenatal steroid treatment
VUE: villitis of unknown aetiology
IVH: occurrence and grade of intraventricular haemorrhage (according to the classification of De Vries et al)
ROP: occurrence and grade of retinopathy of prematurity (according to the International Classification of Retinopathy of Prematurity)
NEC: occurrence and grade of necrotizing enterocolitis (according to the criteria of Bell),
RDS: occurrence and grade of respiratory distress syndrome (according to the classification of Giedion et al)
NS: non significant

Table 4 Predictors for neurodevelopmental outcome

Predictors	Abnormal development at 2 years of age OR (95% CI)
GA	NS
BW	0.7 (0.6-0.9)
UA absent/reverse	NS
DV abnormal	NS
Apgar 5'<6	NS
UC pH <7.00	5.0 (1.1-23.7)
Male sex	NS
Severe composite morbidity	NS
Abnormal/suspect neurological exam at term	NS
IVH	NS
PVL	NS
VUE	3.5 (1.4-8.5)
NEC	NS
RDS	NS

GA: gestational age
BW: birth weight
UA absent/reverse: umbilical artery absent/reverse end diastolic flow
DV abnormal: pulsatility index of the ductus venosus >2 standard deviations above the GA mean
UC pH: pH of the umbilical artery
IVH: occurrence and grade of intraventricular haemorrhage (according to the classification of De Vries et al)
PVL: occurrence and grade of periventricular leukomalacia (according to the classification of de Vries et al)
VUE: villitis of unknown aetiology
NEC: occurrence and grade of necrotizing enterocolitis (according to the criteria of Bell)
RDS: respiratory distress syndrome

Discussion

To our knowledge, the present study is the first to examine the relationship between VUE and outcome in this specific population. The aetiology of VUE is not understood, but it may be linked to a maternal inflammatory response against the foetal allograft or to an underlying infection (190;191). VUE has been shown to be associated with growth restriction (134;181;192;193) and more recently, two studies reported that VUE is associated with long term neurological deficits in infants delivered at term (194;195). Interestingly, in our preterm IUGR cohort a similar association between placental VUE and abnormal neurodevelopment was found. Furthermore, VUE was also associated with NEC. A Pubmed search yielded no literature on associations between VUE and NEC. However, infants with NEC have been shown to have higher mortality rates and a recent review showed that NEC survivors are at an increased risk for long term neurodevelopmental impairment (196). In the present study, NEC was not independently associated with mortality or neurodevelopmental outcome, indicating that confounding between VUE and NEC was not the case. The pathophysiological basis of the VUE associations found in the present study need to be elucidated in future research. In the mean time, we recommend that placentae from pregnancies complicated by early-onset IUGR should routinely be examined for signs of VUE. Presence of VUE may aid neonatologists in early identification of infants at increased risk of NEC, death and abnormal neurodevelopment.

Long term outcome was not only associated with VUE but was also related to weight at birth which indicates that the severity of IUGR and malnutrition affect long term outcome. Moreover, acidosis at birth was related to outcome. This suggests that delivery may have to occur before foetal acidosis develops. Antenatal heart rate abnormalities are related to hypoxemia (preceding acidemia) (180;197;198) suggesting that abnormal FHR should be an indication for delivery. This is currently being studied in the TRUFFLE trial (*T*rial of *U*mbilical and *F*oetal *FL*ow in *E*urope) in which computerised FHR variation and ductus venosus flow velocity waveform patterns are being studied as indicators of foetal impairment and delivery (199). Interestingly, there were no associations between neurodevelopmental outcome and IVH, PVL, neurological examination at term or severe neonatal complications. It has been well established that serious abnormalities on cranial ultrasound are strongly associated with cerebral palsy and delayed mental development (183). A possible explanation for the lack of association between IVH or PVL and neurodevelopmental outcome in the present study, is the fact that no infants had severe IVH (grade 3 or 4) or severe PVL (grade 2 or more). Resnick and colleagues have shown that adverse perinatal conditions lead to severe educational disabilities, but

that less severe disabilities are more influenced by sociodemographic factors (200). It is possible that in the present study genetic factors, socioeconomical status and/or parental educational level were also of importance in determining long term outcome. This theory is supported by the finding that variables significantly associated with neurodevelopment at 2 years of age accounted for only 22% of abnormal development. Unfortunately, due to the study design, we were unable to trace socioeconomical or parental educational level information for this cohort and due to small numbers no stratification into mild or severe developmental delay was possible. Another limitation due to study design is the possibility of selection bias. However, our study group was made homogeneous by the fact that strict criteria were used to select the study population and by the fact that all data are from a single tertiary referral centre where the same criteria and same protocols were used to diagnose and manage the various disease states. Importantly, in the present study multivariate analysis without categorization was used to determine which antenatal variables were of predictive value for outcome. Categorizing continuous variables may lead to loss of important information, because for instance infants with a GA of 26 weeks are considered to be equal to those with a GA of 28 weeks.

Interestingly, in this group of IUGR foetuses antenatal steroid treatment was not significantly related to any of the neonatal outcomes, including RDS. It has been postulated that IUGR may enhance foetal lung maturation (24), but to date no clinical studies specifically studying IUGR foetuses have been able to confirm that clinical respiratory outcome is better (25-27;100). However, a recent study by our group shows that the lecithin/sphingomyelin (L/S) ratio is in fact higher in IUGR foetuses, indicating that biochemical measures of lung maturation are increased when placental insufficiency is present (157). Exogenous administration of glucocorticoids may therefore be of little additional benefit in IUGR foetuses.

As expected, the present study confirms results from previous studies with GA (38-40;46-48) and birth weight (39;41;42;45;46;48) as the most important predictors for mortality and morbidity. Furthermore, also in line with earlier reports, umbilical artery (26;39;41;43) and ductus venosus (38-41;44;47) Doppler indices were significantly associated with outcome. It is important to realise, however, that the variables studied in the present study account for merely 40% of overall death and 43% of severe neonatal complications, which is in line with the recent report by Baschat et al (39). As mentioned by this group, other additional antenatal predictors may need to be considered in future research. For clinical practice, it would be valuable if a prognostic model could be developed to counsel parents as to outcome. Unfortunately the current study was too small to do so in this population. Hopefully, the ongoing TRUFFLE study will shed more light on the optimal timing of delivery of these compromised foetuses (199).

In summary, the present study confirms that GA, birth weight and Doppler findings are important predictors for neonatal outcome in growth restricted foetuses. In addition, this study shows that the presence of placental VUE may aid neonatologists in early identification of infants at increased risk of NEC, death and abnormal neurodevelopment. Abnormal neurodevelopment at follow-up was related to birth weight and acidosis at birth indicating that the severity of malnutrition and foetal acidosis affect long term outcome. Mortality decreased with increasing gestational age, but abnormal neurodevelopment continued to be high, involving approximately 20% of infants born at the various ages. The GRIT and other studies have shown that gestational age is the most important variable determining short term outcome before 30 weeks of gestation. Our study suggests that delivery of infants born after 30 weeks may have been too late in some cases, exposing them too long to continuing undernutrition.

Acknowledgements

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PART 2

PHARMACOLOGICAL TREATMENT OF HYPOXIC-ISCHEMIC REPERFUSION INJURY OF THE NEWBORN BRAIN IN THE CLINICAL SETTING; WHAT ARE THE POSSIBILITIES IN THE NEAR FUTURE?

9

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Abstract

Despite ongoing research and new insights into the pathophysiological pathways of neonatal hypoxic-ischemic reperfusion brain injury, effective treatment is not yet available. This review briefly covers the mechanisms of hypoxic-ischemic reperfusion injury and focuses on promising pharmacological neuroprotective strategies which may become available for clinical use in the near future. Furthermore, the beneficial effects of early intervention and combination therapy with moderate hypothermia are discussed.

Introduction

Birth asphyxia is an important cause of perinatal mortality and lifelong neurodevelopmental morbidity, including cerebral palsy, learning disabilities and mental retardation (4;6;7). There is increasing evidence that a substantial amount of damage occurs upon and up to days after reperfusion and reoxygenation (201;202). This reperfusion injury is triggered by a cascade of biochemical alterations (Fig. 1). Intervention in these destructive pathways may ameliorate delayed cerebral brain damage due to perinatal hypoxia-ischemia. Moderate hypothermia of the newborn brain has been proposed to reduce reperfusion injury after severe perinatal hypoxic-ischemia (203). It is probable that protection can be improved by combining hypothermia with pharmacological means of protection.
The purpose of this review is to discuss the most important biochemical pathways leading to delayed cell death after severe perinatal hypoxia-ischemia and to indicate the possibilities of pharmacological intervention in these pathways in order to reduce or prevent delayed reperfusion injury of the brain. Finally, combination of therapies will be discussed briefly, including moderate hypothermia, in order to achieve optimal neuroprotection against reperfusion injury of the immature brain after severe birth asphyxia.

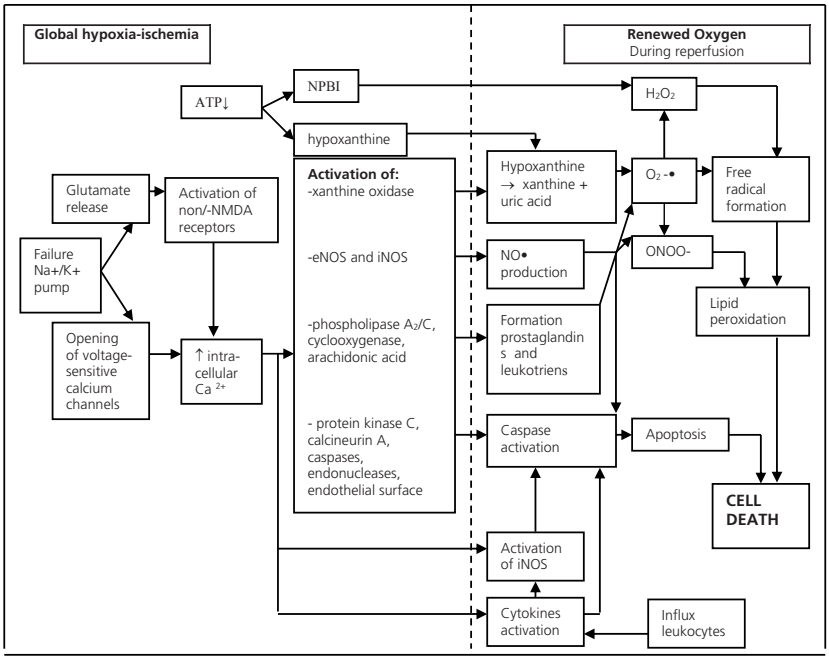

Figure 1 Biochemical pathways leading to delayed neuronal cell death

Mechanisms of hypoxic-ischemic reperfusion injury in the neonate

The neonatal brain contains high concentrations of fatty acids, low concentrations of antioxidants, a high rate of oxygen consumption and available iron. With these characteristics the brain is vulnerable to oxidative damage (15;204). During hypoxia-ischemia there is a decreased delivery of oxygen and glucose to the brain with a consequent depletion of ATP and failure of the Na^+/K^+ ATP-dependent pump at the neural membrane leading to depolarization of the neural membrane and cytotoxic oedema (201;202). These changes in cell homeostasis cause neuronal injury from excitatory amino acids, intracellular calcium accumulation, free radical generation (through xanthine oxidase (XO) and non-protein bound iron (NPBI), nitric oxide synthase (NOS), and cyclooxygenase (COX) and lipooxygenase) and pro-inflammatory cytokines (17;202;205) leading to programmed neuronal cell death (65). However, perinatal hypoxia-ischemia might also alter levels of endogenous neuroprotective factors in neuronal cells (e.g. hypoxia inducible factor-1α (HIF-1α) (206;207) and insulin-like growth factor (IGF) (208)).

Free radical formation

Although several biochemical pathways lead to delayed neuronal cell death (as illustrated in Fig. 1), this paper will mainly discuss the relationship between perinatal hypoxia-ischemia and free radical induced reperfusion injury of the brain.

Free oxygen radicals are reactive compounds with an uneven number of electrons in their orbit. This makes them unstable. Reactive oxygen species (ROS) donate or take electrons from other molecules to pair their electrons and generate stability. This means they can react with normal cellular compounds (fatty acids of membrane lipids or DNA) leading to irreversible damage. Small amounts of ROS are very commonly formed during normal metabolism and are scavenged by the body's anti-oxidants. However, during hypoxia-ischemia and upon reperfusion the limited endogenous anti-oxidative capacity of the newborn brain is overwhelmed by greatly increased production of ROS. This results in 'oxidative stress' (209;210) and consequent cell damage. Reactions involving free radicals have a high potential in neonatal brain, because fast growing tissues are especially sensitive to free radicals. ROS are generated from different sources and in this review we will focus on the three most important pathways leading to ROS production. Pharmacological intervention in these pathways may lead to neuroprotection.

First, acute production of the superoxide free radical ($O_2^{\bullet -}$, see also below) and waterperoxide (H_2O_2) after severe birth asphyxia may promote the formation of the extremely toxic hydroxyl radical (OH•) through the Fenton (or Haber-Weiss) reaction (15). This reaction is catalyzed by NPBI (15;204), released from its binding-protein under hypoxic-ischemic conditions (17). Second, the activation of neuronal and inducible NOS (nNOS and iNOS respectively, see also below) leads to the generation of the nitric oxide radical (NO•) that can react with superoxide to form the toxic peroxynitrite ($ONOO^-$) (205). Peroxynitrite and ROS can damage DNA, lipids and proteins leading to permanent damage of the neuron and cell death. Finally, hypoxanthine, accumulated during the hypoxic-ischemic episode as a degradation product of ATP, is metabolised to uric acid by XO (17;201). This reaction gives rise to further formation of superoxide radicals. All these toxic free radicals contribute substantially to reperfusion injury of the brain after severe hypoxia-ischemia.

Early identification of babies at high risk for brain damage due to hypoxic-ischemic reperfusion injury is necessary for the development of effective neuroprotective strategies. Indirect markers of increased ROS production and perinatal brain injury have recently emerged consisting of increased advanced oxidative protein products (AOPP) and NPBI in erythrocytes and plasma of hypoxic newborns (211-213). Research by Dorrepaal et al (213) showed that levels of NPBI in plasma after birth asphyxia were related to the degree of injury and neurodevelopmental outcome. Buonocore et al (11) have shown NPBI to be the most reliable early indicator of intrauterine oxidative stress. They showed 100% specificity and 100% sensitivity for favourable neurodevelopmental outcome at 0-1.16 umol/l and for poor neurodevelopmental outcome at values >15.2 umol/l. In contrast the results from this study for AOPP were not significant. The study also reports that the nucleated red blood cell (NRBC) count at birth is a reliable predictive marker. Earlier reports have also shown increased NRBC at birth to be a marker of adverse outcome after perinatal hypoxia-ischemia, as this marker was significantly higher in neonates with long-term sequelae than in neonates with the same clinical condition at birth but with normal neurological development (214;215). Early markers of intrauterine oxidative stress, including NPBI and NRBC, may improve the ability of clinicians to identify babies at risk of developing neurodisability.

Clinical pharmacological intervention may be possible, since the free iron chelator deferoxamine, erythropoietin, selective NOS-inhibitors and the XO inhibitor allopurinol are all pharmacological compounds which have shown beneficial effects in animal (and human) studies.

Deferoxamine

Deferoxamine has several mechanisms of action in the prevention of hypoxic-ischemic brain injury. Figure 2 provides an overview of the neuroprotective properties of deferoxamine. First, it binds NBPI (215;216) preventing the formation of the hydroxyl radical (15). Furthermore, deferoxamine prevents the metal-catalyzed nitration of peroxynitrite (217) and it may modulate neutrophil adhesive functions, reducing the inflammatory response (218). Additionally, and perhaps most importantly, deferoxamine may upregulate protective genes against hypoxia-ischemia by stabilization of HIF-1α in the brain (206;219). This factor is activated by various stressors, for example hypoxia (220;221), hypoglycaemia and oxidative stress**.** HIF-1α is a transcription factor and is responsible for the activation of a set of hypoxic-ischemic inducible genes (206;222-224) giving rise to the production of important proteins such as erythropoietin and vascular endothelial growth factor (VEGF) and glycolytic enzymes. These proteins induce adaptive processes such as angiogenesis, anti-apoptotis and stimulation of growth factors (220). Previous studies in hypoxic-ischemic animals has shown that rescue treatment with deferoxamine preserved cerebral oxygen utilization and neuronal integrity and had a positive effect on neurophysiological recovery. These observations indicate a reduction of hypoxic-ischemic brain injury and a more favourable neurodevelopmental outcome (73;206;215). Earlier reports (216) have noted adverse effects in premature baboons, including acute haemodynamic side-effects after intravenous infusion of deferoxamine, however, much higher dosages (continuous infusion of 1,25-10 mg/kg/h during 12-42 hours) were used than necessary for the beneficial effects reported above (one single dose of 2.5-200 mg/kg). The results from animal studies that used lower doses of deferoxamine showed prevention of the formation of cerebral oedema, preservation of the cerebral energy status and varying amounts (up to 56%) of neuroprotection of brain cells without adverse effects (73;206;215). This suggests that deferoxamine can be considered a good candidate for clinical trials. Although animal studies seem promising, no human studies in perinatally asphyxiated neonates have yet been performed.

Erythropoietin

Erythropoietin (EPO) was first characterised as a haematopoietic growth factor. As mentioned above, EPO gene expression in the brain is upregulated by HIF-1α stabilization (70;220;221;225-227). The mechanisms through which EPO is thought to exert its effect include reduction of the inflammatory infiltrate (228), reduction of apoptosis

(70;225;226;229), promotion of angiogenesis (230) and neurotrophic activity (226;231). However, the endogenous protective mechanisms of EPO are probably insufficient upon acute injury. It is possible that the brain does not produce enough EPO after cerebral ischemia, or that the latency of neosynthesis is too long to sufficiently protect neuronal tissue. Stimulation by deferoxamine and/or exogenous EPO-administration may achieve EPO's optimal neuroprotective action. Several animal studies have shown that EPO prevents neuronal damage (70;226;229), also when administered after the hypoxic-ischemic insult (69;225;228;232).

Many children and premature newborns (231) have been treated with EPO in recent years, mostly for anaemia. EPO has been shown to be a clinically well-tolerated drug without side effects. EPO seems a promising drug in the reduction and prevention of hypoxic-ischemic induced reperfusion brain injury after severe birth asphyxia. However, the optimal dose and the therapeutic window have not yet been defined and extensive experimental and clinical studies seem warranted.

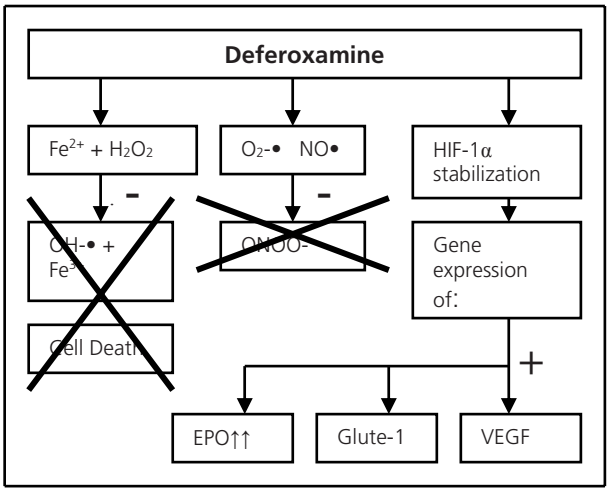

Figure 2 Neuroprotective activity of Deferoxamine

Nitric oxide synthase inhibition

Nitric oxide (NO) is produced by NOS. Three different isoforms of NOS have been identified: the endothelial (eNOS), neuronal (nNOS), both constitutionally present, and inducible NOS (iNOS). NO produced by eNOS (which is localized in the endothelium) is thought to be neuroprotective, as it probably maintains cerebral blood flow by causing

vasodilatation after hypoxia-ischemia (17;233). All NOS-isoforms are upregulated after hypoxia-ischemia, however, in different time profiles during reperfusion (17;202;234). nNOS and eNOS are activated early in the process of hypoxia-ischemia by increased influx of calcium in neuronal and glial cells (202;234). In the brain iNOS can be induced in microglia, astrocytes and endothelial cells and occurs later during upregulation of the cytokine/chemokine pathway (17;202;234) (Figure 3). NO• produced by iNOS and nNOS is an important initiator of neuronal damage through reaction with superoxide to form peroxynitrite (17;201;202). The neuroprotective effect of nNOS and iNOS inhibition can be obtained by a reduced production of peroxynitrite preventing nitrotyrosination of important proteins (65) and selective nNOS and iNOS inhibition seems beneficial in experimental studies (62-66;235-237). 2-iminobiotin (2-IB), a selective inhibitor of nNOS and iNOS developed by our group, prevented development of secondary energy failure immediately after reperfusion and reoxygenation by improving the cerebral energy state (63;237), preventing formation of vasogenic oedema (63) and reducing apoptosis-related parameters such as caspase-3 activity in experimental studies (62;65). Furthermore, 2-IB also improved long-term outcome in 12-day old rat pups at 6 weeks after HI (64;65). Further studies to rule out species specific or developmental effects are warranted. We are currently in the process of further development of 2-IB as a clinical drug.

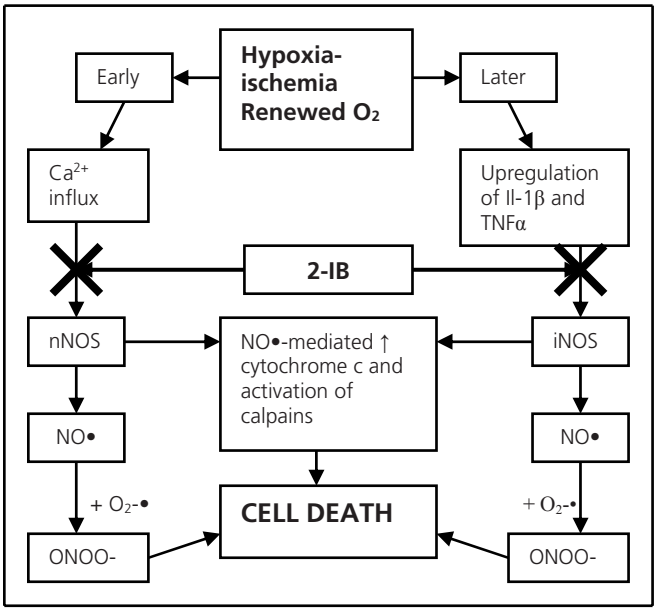

Figure 3 Effect of combined inhibition of nNOS and iNOS with 2-iminobiotin

Xanthine oxidase inhibition with allopurinol

During the hypoxic-ischemic phase tissue stores of ATP are degraded to hypoxanthine and XO is produced from xanthine dehydrogenase by calcium-activated proteases (19). Upon reperfusion and reoxygenation XO promotes the transformation of hypoxanthine into uric acid leading to the production of ROS (15;201).

XO activity can be blocked by allopurinol, a XO inhibitor (238). Other beneficial effects of allopurinol are a direct radical scavenging potential (239) and chelation of NPBI (76;215), but these properties are only exerted when high dosages of allopurinol are used (239) (Fig. 4). In the body allopurinol is converted to oxypurinol, an even better hydroxyl-radical scavenger (239).

Several trials have been performed in asphyxiated neonates (76;238) in which no adverse effects of postnatal administration of allopurinol were detected. Russell (238) et al were unable to prove neuroprotective effects in preterm infants treated with allopurinol. Van Bel et al (76) were able to show that allopurinol treatment reduced free radical formation and maintained cerebral blood flow and electrical brain activity in a small pilot study in severely birth asphyxiated term newborns. Follow-up of this small patient population showed a tendency to a lower mortality in the allopurinol-group, but reduction of mortality did not result in increased morbidity (240). This suggests that allopurinol may exert long-term neuroprotection by reducing both mortality and morbidity. An interim-analysis of a recent multicentre clinical trial, however, did not show any beneficial effect of postnatal allopurinol in reducing post hypoxic-ischemic reperfusion injury of the brain (77). The reason for this lack of effect may be the late point in time of allopurinol administration (median time 3 to 4 hours after birth). At this point the XO-induced superoxide-surge and NPBI-induced hydroxyl-production have already taken place. Neuroprotective effects can be expected from allopurinol if treatment is established earlier. Ideally, allopurinol should be given as soon as the actual hypoxic-ischemic insult is detected and during the early reperfusion phase. Therefore, it would be better to treat the pregnant mother when hypoxia-ischemia is imminent. In this context the results of a study by Boda et al (78) are interesting. They report that allopurinol, when given to pregnant women, shows a rapid therapeutic increase in allopurinol concentrations in the foetus and in plasma of the newborn baby. This study showed no adverse effects of allopurinol in mother or infant.

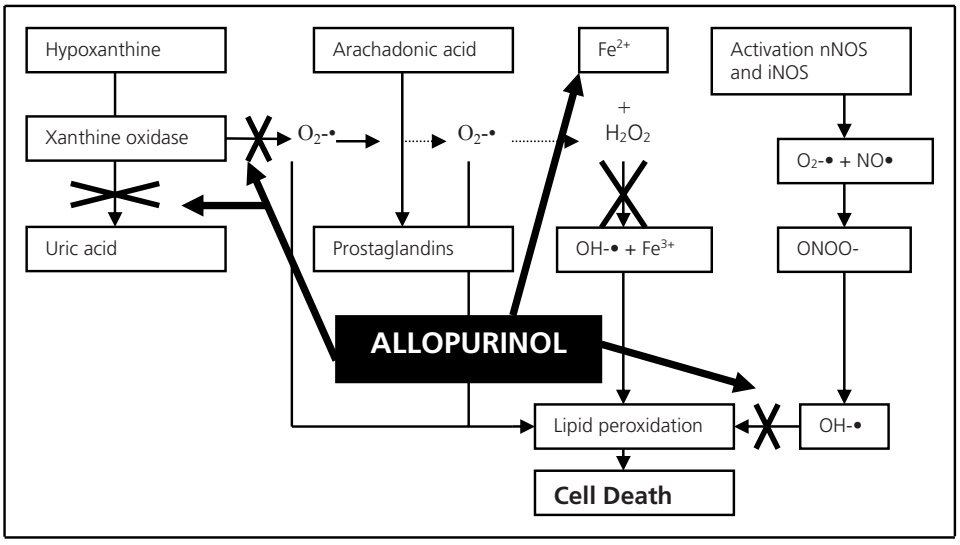

Figure 4 Neuroprotective properties of allopurinol

Hypothermia

Moderate hypothermia has been researched in various animal (241-244) and human studies (60;203;245-250). It is thought that moderate hypothermia prevents brain damage by reduction of caspase activity (241), glutamate-increase and free radical formation (202;244). Selective head cooling combined with mild systemic hypothermia has been proven to be safe in human pilot studies (203;246;248). Tendency towards better outcomes for cooled infants with moderate to severe encephalopathy were reported in one study (246). The recently published study by Gluckman et al (60) reports that selective head cooling combined with mild systemic hypothermia is not protective in a mixed population of infants with neonatal encephalopathy. However, this treatment safely improved survival without severe neurodevelopmental disability in infants with less severe aEEG changes.

For cooling of deep brain structures, the best method remains undefined. An animal study (251) showed that significant reduction in brain temperature could only be achieved by lowering the core temperature. Whole-body cooling may form an appropriate approach, however, the adverse effects of this method may limit its applicability. In recent years three pilot studies have been performed using whole body cooling (245;247;249). Adverse effects that were noticed include; pulmonary hypertension, hypotension, hypoxaemia, bradycardia, thrombocytopenia, disseminated intravascular coagulation

and necrotic skin lesions. However, no life threatening events were recorded. One group studied the outcome of cooled infants and they reported a 52% survival with a normal MRI compared to controls (247). Also, the recently preliminary published data of a randomised trial of systemic hypothermia showed selective protection of the cortex on MRI in term hypoxic-ischemic encephalopathy (250). Hypothermia is probably most effective when it is applied as soon as possible after birth. Current management should not be altered before results from large randomised controlled trials are available. A combination of moderate hypothermia with pharmacological intervention may lead to a further improvement of the clinical outcome.

Combining intervention strategies

In recent years a lot of progress has been made in understanding the pathophysiological pathways of hypoxic-ischemic reperfusion injury. As stated earlier in this review, it is known that a substantial amount of the brain damage in asphyxiated newborns occurs upon and after the reperfusion phase and can last for days (201;202). This fact provides us with a therapeutical window during which intervention can take place. Different pathways related to neuronal cell death or neuronal protection after hypoxia-ischemia have been described (15;17;201;202;204;205). Blocking only one pathway will probably leave the neuron in an activated state, because other biochemical pathways are still intact. Therefore, intervention in various pathways will probably be necessary to improve neuroprotection. Combination therapy seems to be a promising strategy to optimise neuroprotection.

There is some evidence of combination therapies from animal models (241-243). These studies show that combination therapy can work synergistically to reduce hypoxic-ischemic brain injury. So far no human clinical trials have been performed using combination therapy, though this seems a promising approach.

Another major issue is the timing of the intervention. Hypoxic-ischemic reperfusion injury commences in utero, which means that postnatal interventions, are often started too late to provide neuroprotection. We postulate that effective neuroprotection may be achieved when early and combined treatment is used. Figure 5 summarises the treatment proposal. Antenatal allopurinol treatment should be started as soon as intrauterine hypoxia-ischemia is detected. Moderate hypothermia should be installed directly after birth and thereafter deferoxamine should be administered. Furthermore, the activity of nNOS and iNOS should be reduced as soon as possible after birth through selective inhibition. EPO will be formed via stabilization of HIF-1α by deferoxamine, but

the endogenous production will probably be insufficient to exert optimal effects on the longer term. It seems appropriate to supply EPO later in the post-hypoxic-ischemic phase, as its anti-apoptotic and trophic actions are needed during this period.

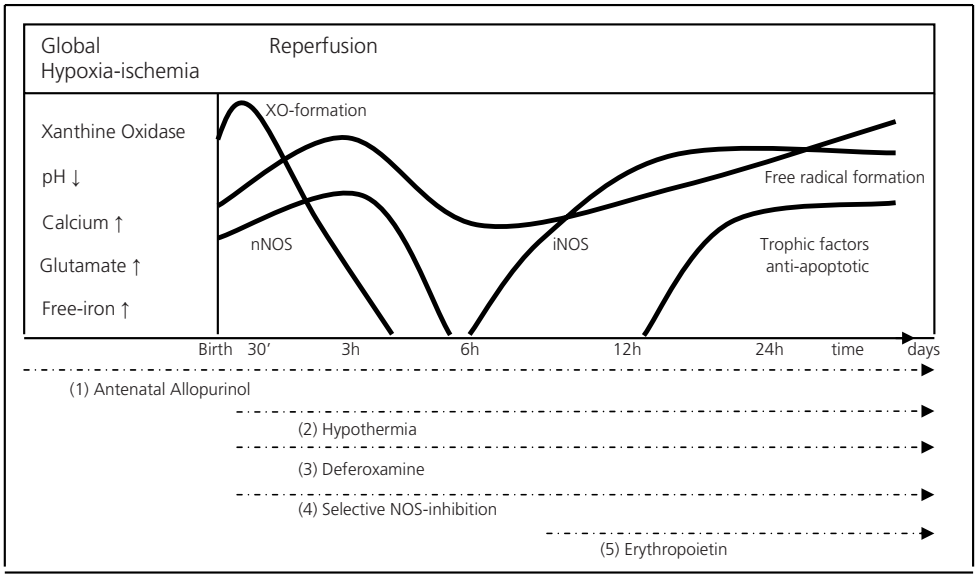

Figure 5 Timing of intervention strategies

In conclusion, pharmacological intervention in important biochemical pathways leading to delayed neuronal cell death after severe perinatal hypoxia-ischemia may be possible in the near future. Combining moderate hypothermia and pharmacological intervention may achieve the most optimal results. Further research along these lines seems warranted.

10

ALLOPURINOL PROTECTS THE UMBILICAL CIRCULATION FOLLOWING REPEATED EPISODES OF ISCHEMIA-REPERFUSION IN THE FOETUS

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Abstract

Background

In complicated labour, neonatal outcome may depend not only on the extent of foetal asphyxia and acidosis, but also on the effects on the foetal cardiovascular system of reactive oxygen species (ROS) generated during the ischemia-reperfusion (I/R) associated with repeated compressions of the umbilical cord.

Objectives

This study tested the inter-related hypotheses that I/R-induced ROS generation will restrict umbilical blood flow secondary to activation of the xanthine oxidase pathway, and that maternal treatment with allopurinol will protect against this effect.

Methods

The hypotheses were tested by investigating in vivo, in the chronically-instrumented sheep foetus in late gestation, the effects of maternal treatment with therapeutic doses of allopurinol (n=6) or with vehicle (n=6) on the foetal cardiovascular system during and following episodes of I/R produced by repeated, measured compressions of the umbilical cord.

Results

The data show that maternal treatment with allopurinol improved the recovery of umbilical blood flow and foetal survival following I/R associated with clinically relevant acidemia and repetitive foetal heart rate decelerations.

Conclusion

The data support the hypotheses tested and suggest that maternal treatment with allopurinol may offer plausible clinical intervention in the management of perinatal asphyxia in complicated labour.

Introduction

One of the most common challenges that the foetus experiences during pregnancy, and in particular during the processes of labour and delivery, are periods of asphyxia secondary to compressions of the umbilical cord (252). Perinatal asphyxia may result in marked foetal acidosis with subsequent hypoxic-ischemic encephalopathy, which is predictive of developing cerebral palsy and cognitive disability later in life (253). Therefore, the prevention and management of perinatal asphyxia remain major concerns in obstetric practice today.

Repeated compressions of the umbilical cord not only induce foetal asphyxia and acidosis but also episodes of ischemia-reperfusion (I/R), promoting the generation of reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\cdot-}$) (254). Increased $O_2^{\cdot-}$ decreases the bio-availability of nitric oxide (NO) and, in the vascular endothelium, an increased ratio of $O_2^{\cdot-}$: NO leads to endothelial dysfunction and vasoconstriction (255). Free radical generation as a result of I/R may therefore restrict blood flow in circulations, particularly in those, which are highly dependent on NO, such as the umbilical vascular bed (256). Hence, free radical-induced changes in umbilical haemodynamics will worsen the effects on the foetus of asphyxia and acidosis resulting from repeated compressions of the umbilical cord.

One mechanism via which ROS are generated is through the activation of the xanthine oxidase (XO) pathway (257). The XO inhibitor allopurinol can offer protection against I/R-induced injury not only by reducing the formation of $O_2^{\cdot-}$ (258), but also through direct free radical scavenging properties and chelation of non protein bound iron (76). Consequently, the beneficial effects of allopurinol in reducing I/R damage in adult cardiology and in paediatric and adult cardiothoracic surgery have long been established (259-261). Treatment with allopurinol of the asphyxiated neonate improved neonatal outcome (76), however, if the time-interval between I/R and treatment had been too long, or when asphyxia had been too severe, no reduction in serious morbidity or mortality was reported (77). Therefore, perinatal outcome may be improved if treatment with allopurinol is initiated during the actual period of I/R, for instance during labour complicated by recurrent umbilical cord compression. Maternal treatment with allopurinol crosses the placenta, it suppresses $O_2^{\cdot-}$ production in the foetus (262) and yields therapeutic levels in the neonatal circulation (78), justifying this route of administration for preventive treatment. However the effects of allopurinol on the foetal cardiovascular system in normal or compromised pregnancy are completely unknown.

This study therefore tested the inter-related hypotheses that I/R-induced free radical generation will restrict umbilical blood flow secondary to activation of the XO pathway,

and that maternal treatment with allopurinol will protect against this effect. The hypotheses were tested by investigating in vivo, in the chronically-instrumented ovine foetus in late gestation, the effects of maternal treatment with therapeutic doses of allopurinol on the foetal cardiovascular system during and following episodes of I/R produced by repeated, measured compression of the umbilical cord.

Methods

Surgical Preparation

All procedures were performed under the UK Animals (Scientific procedures) Act 1986 and were approved by the Ethical Review Committee of the University of Cambridge. Twelve Welsh Mountain Sheep were surgically instrumented for long term recording at 124 days of gestation (term is ca. 145 days) using strict aseptic conditions as previously described in detail (263). In brief, food, but not water, was withheld from the pregnant ewes 24 h prior to surgery. Following induction with 20 $mg.kg^{-1}$ i.v. sodium thiopentone (Intraval Sodium; Merial Animal Health Ltd, Rhone Merieux, Dublin, Ireland), general anaesthesia (1.5-2.0% halothane in 50:50 O_2:N_2O) was maintained using positive pressure ventilation. Midline abdominal and uterine incisions were made, the foetal hind limbs were exteriorised and, on one side, foetal arterial (i.d., 0.86 mm; o.d., 1.52 mm; Critchly Electrical Products, NSW, Australia) and venous (i.d., 0.56 mm; o.d., 0.96 mm) catheters were inserted. The catheter tips were advanced carefully to the descending aorta and inferior vena cava, respectively. Another catheter was anchored onto the foetal hind limb for recording of the reference amniotic pressure. In addition, a transit-time flow transducer was implanted around the left umbilical artery close to the common umbilical artery inside the foetal abdominal cavity (4SB; Transonic Systems Inc., Ithaca, NY, USA). An inflatable occluder cuff (In Vivo Metrics) was positioned around the proximal end of the umbilical cord, as described previously in detail (263). The uterine incisions were closed in layers, the dead space of the catheters was filled with heparinised saline (80 $i.u.\ heparin.ml^{-1}$ in 0.9% NaCl) and the catheter ends were plugged with sterile brass pins. Ewes were instrumented with arterial and venous catheters placed in the left femoral artery and vein, respectively. The catheters, occluder and flow probe leads were then exteriorised via a keyhole incision in the maternal flank and kept inside a plastic pouch sewn onto the maternal skin.

Post-Operative Care

During recovery, ewes were housed in individual pens in rooms with a 12 h : 12 h / light: dark cycle. Here, they had free access to hay and water and were fed concentrates twice daily (100 g sheep nuts no. 6; H & C Beart Ltd., Kings Lynn, IK). Antibiotics were administered daily to the ewe (0.20-0.25 $mg.kg^{-1}$ i.m. Depocillin; Mycofarm, Cambridge, UK) and foetus i.v. and into the amniotic cavity (150 $mg.kg^{-1}$ Penbritin; SmithKline Beecham Animal Health, Welwyn Garden City, Hertfordshire, UK). The ewes also received 2 days of post-operative analgesia if required (10-20 $mg.kg^{-1}$ oral Phenylbutazone; Equipalozone pate, Arnolds Veterinary Products Ltd., Shropsshire, UK), as assessed by their demeanor and feeding patterns. Generally, normal feeding patterns were restored within 48 h of post-operative recovery. Following at least 72 h of recovery, ewes were transferred to metabolic crates where they maintained for the remainder of the protocol. The arterial and amniotic catheters were connected to sterile pressure transducers (COBE; Argon Division, Maxxim medical, Athens, Texas, USA) and the flow probe lead to a flow meter (T206; Transonic Systems Inc., Ithaca, NY, USA). Whilst on the metabolic crates, the patency of the foetal catheters was maintained by a slow continuous infusion of heparinised saline (80 i.u. $heparin.ml^{-1}$ at 0.1 $ml.h^{-1}$ in 0.9% NaCl) containing antibiotic (1 $mg.ml^{-1}$ benzylpenicillin; Crystapen, Schering-Plough, Animal Health Division, Welwyn Garden City, UK).

Experimental Protocol

Following at least 5 days of post-operative recovery, all foetuses were submitted to an I/R challenge, produced by 5 x 10 min inflations of the cord occluder with sterile saline at 10-minute intervals (Fig. 1). Each cord compression was designed to reduce umbilical blood flow by 80-90% from baseline, and to lead to a progressive fall in foetal arterial pH to 6.9. In 6 foetuses, the I/R challenge was induced during maternal i.v. treatment with allopurinol (Sigma Ltd., 20 $mg.kg^{-1}$ maternal weight, dissolved in buffered saline and infused over a twenty minute period). In the remaining 6 foetuses, the I/R challenge was induced during maternal infusion with buffered saline at the same rate. Infusion of either allopurinol or vehicle started 10 min before the 4th umbilical cord compression and finished immediately after the end of it. The dosing regimen of allopurinol was adopted from the only study that used the drug in women undergoing uncomplicated labour (78). Forty-eight hours after the end of the experimental protocol, ewes and foetuses were subjected to humane euthanasia using a lethal dose of sodium pentobarbitone (200$mg.kg^{-1}$ i.v. Pentoject; Animal Ltd., York, UK). The positions of the implanted catheters, occluder and flow probe were confirmed and the foetus was weighed.

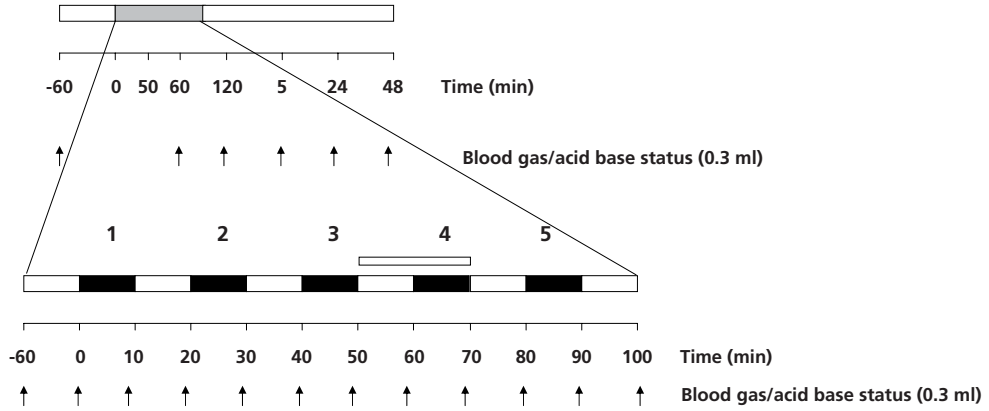

Figure 1 Experimental protocol
At least 5 days after surgery all foetuses were submitted to an I/R challenge (gray bar). Following 1 h of basal recording, the I/R challenge consisted of 5 compressions of the umbilical cord, each of 10 minute duration (black bars) with a 10 minute interval. Maternal infusion with allopurinol or vehicle started 10 min before the 4th umbilical cord compression and finished immediately after the end of it (white bar). Foetal arterial blood samples (arrows) were taken for analysis of blood gas and metabolic status before, during and after each umbilical cord compression. Foetal cardiovascular variables were recorded continuously using a data acquisition system.

Blood sampling regimen

Maternal and foetal arterial blood samples (0.3 ml) were drawn into sterile syringes 1 h prior, at 10 min intervals during, and for 48 h following the I/R challenge to determine arterial blood gas and acid base status (Fig. 1; ABL5 Blood Gas Analyzer, Radiometer, Copenhagen, Denmark; maternal measurements corrected to 38 °C and foetal measurements corrected to 39.5°C). Values for percentage saturation of haemoglobin with oxygen (Sat Hb) were determined using a hemoximeter (OSM3; Radiometer). Blood lactate concentrations were measured by an automated analyser (Yellow Springs 2300 Stat Plus; YSI Ltd., Farnborough, UK). Additional paired maternal and foetal blood samples (1 ml) were taken in the allopurinol treated pregnancies at varying set intervals, starting at the end of the infusion period and up to 6 h following the end of infusion, to compile a comprehensive serial profile of maternal and foetal plasma concentrations of allopurinol and oxypurinol without affecting materno-foetal concentrations of haemoglobin. Reversed-phase high-performance liquid chromatography (HPLC) with UV-detection at 254 nm was used for the quantification of allopurinol and oxypurinol in both foetal and maternal plasma. The method was linear between 0.5 and 25 $mg.l^{-1}$ with a lower limit of detection of 0.2 $mg.l^{-1}$ for both compounds (264).

Data and Statistical Analyses

Mean maternal and foetal (corrected for amniotic pressure) arterial blood pressure, maternal and foetal heart rate (triggered from the arterial pulse) and mean umbilical blood flow were recorded continually at 1 s intervals using a computerised Data Acquisition System (Department of PDN, University of Cambridge, UK). Values for all variables are expressed as mean±S.E.M. Cardiovascular variables are expressed as minute averages of the percent changes from mean baseline. Summary measures analysis was applied to the cardiovascular serial data to focus the number of comparisons and areas under the curve were calculated for statistical comparison, as previously described in detail (265). Variables were assessed using two-way ANOVA with repeated measures (Sigma-Stat; SPSS Inc., Chicago, IL, USA) comparing the effect of time, group and interactions between time and group. Where a significant effect of time or group was indicated, the post hoc Student-Newman-Keuls test was used to isolate the statistical difference. For all comparisons, statistical significance was accepted when $P < 0.05$.

Results

Allopurinol and oxypurinol in maternal and foetal plasma

Maternal treatment with allopurinol produced transient elevations in the concentrations of both allopurinol and oxypurinol in maternal and foetal plasma (Fig. 2). In maternal plasma, peak concentrations of allopurinol and oxypurinol occurred between 20-30 min after the start of infusion, reaching values of 47 and 17 $mg.L^{-1}$, respectively. In foetal plasma, elevations in allopurinol between 4 and 7 $mg.L^{-1}$ were within the therapeutic ranges described by Boda et al. (78) and these were maintained past 1 h after the start of maternal administration. Relative to allopurinol, the elevation in oxypurinol in foetal plasma was more gradual and it lasted much longer, reaching peak values between 1-1.5 $mg.L^{-1}$ at 2 h after the start of infusion.

Maternal variables

Basal maternal arterial blood gas and acid base status were not different between vehicle and allopurinol pregnancies (vehicle group: pH 7.52±0.01, $PaCO_2$ 39±1 mmHg, PaO_2 103±3 mmHg, blood lactate 0.5±0.1 $mmol.L^{-1}$, ABE 7.9±0.9 $mEq.L^{-1}$, SatHb 97±1%; allopurinol group: pH 7.52±0.01, $PaCO_2$ 37±0.4 mmHg, PaO_2 100±4 mmHg, blood lactate 0.4±0.0 $mmol.L^{-1}$, ABE 7.0±0.9 $mEq.L^{-1}$, SatHb 97±1%). These values remained unaffected from baseline throughout the experimental protocol. Basal maternal arterial blood pressure (MAP) and heart rate (MHR) were not different between groups (vehicle:

MAP 104+10 mmHg, MHR 97+6 bpm; allopurinol: MAP 99+5 mmHg, MHR 91+5 bpm). While MAP remained unaffected from baseline in both groups, there was a transient increase from baseline in MHR to a maximum of 156+20 bpm at 52 min following the onset of infusion in ewes treated with allopurinol ($P<0.05$). MHR in allopurinol treated ewes returned to basal values by 109 min after the onset of infusion.

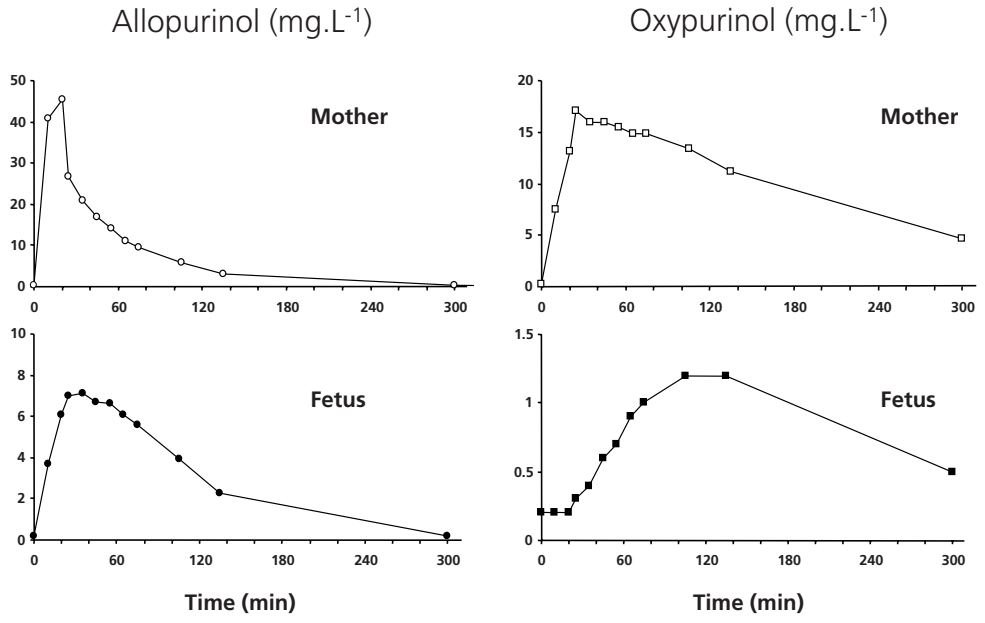

Figure 2 Maternal and foetal plasma concentrations of allopurinol and oxypurinol
The data show the serial profile of plasma concentrations of allopurinol and oxypurinol in maternal and foetal plasma following maternal treatment with allopurinol. Paired maternal and foetal blood samples (1 ml) were taken in the allopurinol-treated pregnancies at varying set intervals, starting at the end of the allopurinol infusion period and up to 5 h following the end of infusion.

Foetal arterial blood gas and metabolic status

Compressions of the umbilical cord produced transient episodes of asphyxia of similar magnitude in both vehicle and allopurinol groups, characterised by transient decreases in foetal PaO_2 and SatHb and transient increases in foetal $PaCO_2$ (Fig. 3). Repeated umbilical cord compression also led to a gradual decline in foetal basal pH and ABE and a gradual increase in foetal basal lactate concentrations. Again, the magnitudes of these changes were similar between vehicle and allopurinol groups (Fig. 3). By the end of the I/R challenge, the changes in variables reflecting arterial blood gas and metabolic status in vehicle and allopurinol pregnancies, respectively, were: foetal pH reduced from 7.36

± 0 at baseline to 6.97 ± 0.03 at the end of the 5th compression vs. 7.36 ± 0 to 6.97 ± 0.03 (both groups P<0.05), ABE from 4.8 ± 0.5 mEq.L^{-1} to -15 ± 1.7 mEq.L^{-1} vs. 3.6 ± 0.8 mEq.L^{-1} to -15 ± 1.3 mEq.L^{-1} (both groups P<0.05), PaO_2 from 21 ± 2 mmHg to 14 ± 1 vs. 21 ± 2 mmHg to 13 ± 2 mmHg (both groups P<0.05), SatHb from 55+5 to 22+2 % vs. 58+7 to 23+6 % (both groups P<0.05), $PaCO_2$ from 58 ± 1 mmHg to 91 ± 6 mmHg vs. 55 ± 2 mmHg to 91 ± 5 mmHg (both groups P<0.05), and blood lactate from 1.1 ± 0.2 mmol.L^{-1} to 9.8 ± 1.7 mmol.L^{-1} vs. 1.1 ± 0.2 mmol.L^{-1} to 9.9 ± 1.2 mmol.L^{-1} (both groups P<0.05).

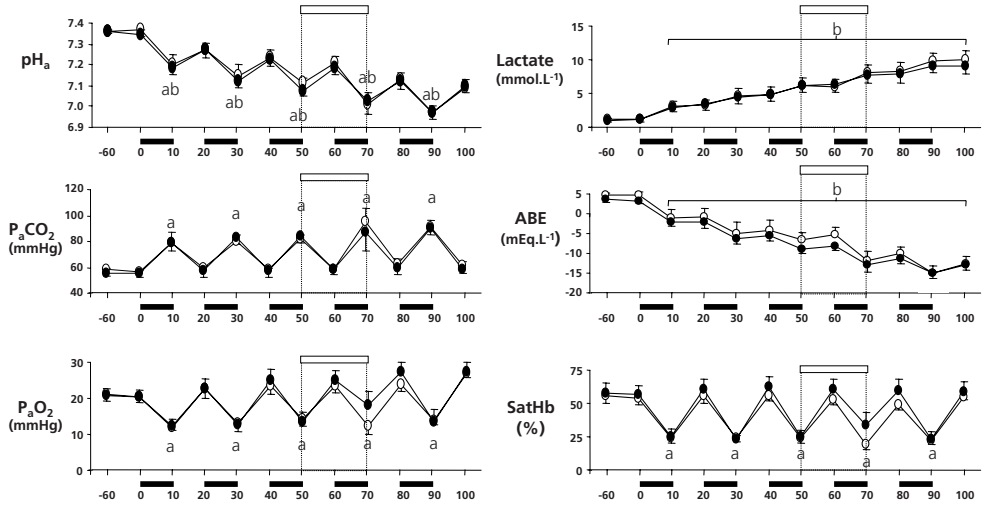

Figure 3 Foetal blood gas and acid base status
Data are the mean±S.E.M. for foetal blood gas and acid base status in samples taken before, during and after each umbilical cord compression. In 6 foetuses (●), the I/R challenge was induced during maternal i.v. treatment with allopurinol (20 mg.kg^{-1} maternal weight, dissolved in buffered saline and infused over a twenty minute period). In the other 6 foetuses (○), the I/R challenge was induced during maternal infusion with buffered saline at the same rate. Infusion of either allopurinol or vehicle started 10 min before the 4th umbilical cord compression and finished immediately after the end of it (white bar). There were no significant differences between groups. For both groups, significant differences within groups are: $^{a}P < 0.05$ *vs.* immediately preceding baseline; $^{b}P < 0.05$ *vs.* -60 minute sample (Two-way RM ANOVA with post hoc Student Newman Keuls test). pHa, arterial pH; $PaCO_2$, arterial partial pressure of CO_2; PaO_2, arterial partial pressure of O_2; Lactate, blood lactate concentration; ABE, acid base excess; SatHb, percentage saturation of haemoglobin with oxygen.

Foetal arterial blood pressure and foetal heart rate responses

Mean values for basal foetal arterial blood pressure and basal foetal heart rate were similar between vehicle and allopurinol groups (55.4 ± 3.6 mmHg vs. 53.8 ± 3.6 and 164 ± 5 vs. 163 ± 10 bpm, respectively). Values for these variables during the I/R experimental protocol are shown as percentage changes from baseline in Figure 4. Compression of

the umbilical cord led to rapid transient falls in foetal heart rate and rapid transient increases in foetal arterial blood pressure. The magnitude and pattern of these changes were similar between vehicle and allopurinol pregnancies until the onset of maternal treatment with allopurinol. Maternal treatment with allopurinol led to an increase in values for foetal heart rate calculated before the fourth (185 ± 17 vs. 193 ± 13 bpm) and fifth (193 ± 14 vs. 210 ± 12 bpm) compression (control vs. allopurinol, area under the curve, $P<0.05$). Following the I/R challenge, foetal arterial blood pressure remained significantly below, and foetal heart rate remained significantly above, basal values in both groups of pregnancies until the end of the 5 h recording period (Fig. 4). The pattern and magnitude of the fall in foetal arterial blood pressure post-I/R were similar between the groups. In contrast, post-I/R, while the increase in foetal heart rate was mild and sustained in control pregnancies, it was markedly and transiently exacerbated in allopurinol pregnancies, showing a 53 % increase and reaching a maximum value of 246 ± 15 bpm at 110 min after the onset of maternal treatment with allopurinol.

Umbilical haemodynamics

Mean values for basal umbilical blood flow were similar between vehicle and allopurinol groups (138 ± 19 vs. 135 ± 28 $ml.min^{-1}$, respectively). Values for umbilical blood flow during the experimental protocol are shown as percentage changes from baseline in Figure 4. Umbilical blood flow was similarly reduced by 80-90% from baseline during each compression period in both vehicle and allopurinol pregnancies. After the end of the I/R challenge, umbilical blood flow returned to basal values within 5 minutes in pregnancies treated with allopurinol. In marked contrast, in pregnancies treated with vehicle, umbilical blood flow remained significantly depressed from basal values until the end of the recording period, 5 hours after the start of the challenge (Fig. 4). This difference between the groups persisted whether the data were expressed as changes in umbilical blood flow, umbilical vascular resistance or umbilical vascular conductance (data not shown).

Foetal survival

All foetuses in the pregnancies treated with allopurinol survived the I/R challenge and they were in good physiological condition 48 hours after the end of the study protocol, with values representing foetal arterial blood gas and metabolic status similar to those measured prior to I/R. In contrast, one foetus from the vehicle treated pregnancies died 3 hours after the end of the I/R challenge as a result of cardiovascular collapse. Although in this foetus values representing arterial blood gases and metabolic status at

the end of the fifth compression period were comparable to all other surviving foetuses in this group (pH = 6.99, $PaCO_2$ = 91 mmHg, PaO_2 = 17 mmHg, SatHb = 30%, ABE = -14 $mEq.L^{-1}$, blood lactate = 12 $mmol.L^{-1}$), the foetus was not able to maintain blood pressure after the experiment. Values for cardiovascular data in this foetus after the end of the I/R challenge have been excluded from the data analysis.

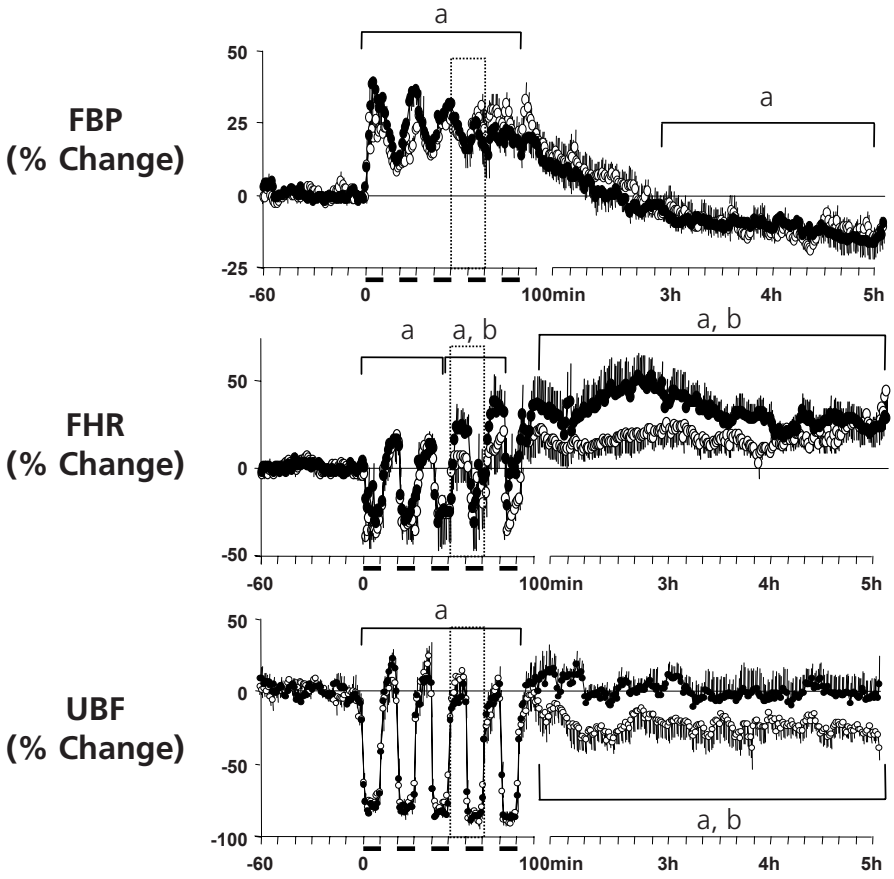

Figure 4 Foetal cardiovascular variables
Data are the mean±S.E.M. of the percent change from mean baseline calculated every minute for foetal arterial blood pressure (FBP), foetal heart rate (FHR), and umbilical blood flow (UBF) in 6 pregnancies which received maternal i.v. treatment with allopurinol (●) and in 6 pregnancies which received maternal i.v. treatment with buffered saline (○) during the the I/R challenge. Infusion of either allopurinol or vehicle started 10 min before the 4th umbilical cord compression and finished immediately after the end of it (dotted box). Significant differences are comparisons of area under the curve: $^{a}P < 0.05$ vs. baseline 1 h period; $^{b}P < 0.05$, allopurinol *vs.* saline (Two-way RM ANOVA with post hoc Student Newman Keuls test).

Discussion

In obstetric practice today repetitive decelerations in foetal heart rate coupled with a significant reduction in foetal pH signals complicated labour (266), which usually leads to clinical intervention to protect the unborn child and thereby improve neonatal outcome (267). Common current clinical interventions in complicated labour include inhibition of uterine contractions, electing for Caesarean section or performing instrumental vaginal delivery. More recently, it has been investigated whether treatment with the antioxidant allopurinol of severely asphyxic human neonates could protect their brain against ischemia-reperfusion damage (76;77;268). While treatment with allopurinol of severely asphyxic human neonates reduced free radical formation and improved cerebral perfusion and electrocortical activity (76), allopurinol treatment started postnatally was deemed too late to prevent brain damage (77). This has led to the suggestion that antioxidant therapy in complicated labour should be brought forward and to treat the mother rather than the offspring. Consequently, it has been proposed that maternal treatment with allopurinol should commence upon suspicion of foetal distress during labour (77). Therefore, the first aim of this investigation was to create an animal model in which the effects of allopurinol on maternal and foetal health could be investigated mimicking the clinical situation of labour complicated by repeated compression of the umbilical cord, leading to repetitive foetal heart rate decelerations and clinically relevant foetal acidemia.

The data show that in the late gestation ovine foetus repeated, intermittent, measured compression of the umbilical cord designed to reduce umbilical blood flow by 80-90% of basal values led to repeated foetal bradycardia and progressive foetal acidemia of human clinical relevance (mean+SEM: 6.97 ± 0.03 in both vehicle and allopurinol treated pregnancies). Maternal treatment with allopurinol during established foetal academia led to rapid increases in maternal and foetal plasma concentrations of allopurinol. In foetal plasma, elevations in allopurinol between 4 and 7 $mg.L^{-1}$ were achieved within 20 minutes of maternal administration, a range that is within the therapeutic levels described by Boda et al. (78). The fact that therapeutic levels of allopurinol in foetal plasma are rapidly achieved further supports its use in the treatment of acute foetal asphyxia during labour**.**

Following the I/R challenge, umbilical blood flow in untreated pregnancies remained significantly depressed from basal values until the end of the recording period, 5 hours after the start of the challenge. In addition, one foetus in this group died 3 hours after the end of the I/R challenge as a result of cardiovascular collapse. In marked contrast, in pregnancies treated with allopurinol, umbilical blood flow was restored to basal values

within 5 minutes following I/R and all foetuses in this group survived the challenge. These data suggest that I/R-induced increases in the ratio of vascular $O_2^{\cdot-}$: NO worsens the effects on the foetus of asphyxia and acidosis by restricting umbilical blood flow to extents that may lead to foetal cardiovascular collapse and death. Further, the data show that the physiologic mechanism underlying the oxidant effects of I/R on the umbilical vascular bed is due to activation of the XO pathway. At least three observations in the literature are consistent with these findings. First, XO is known to be present in the human placental vascular bed (269). Second, placental XO activity is upregulated during labour, augmenting the production of free radicals at the foeto-placental interface (270). Third, it has been reported that ROS, generated by hypoxanthine and XO, potentiated the vascular tension in the human umbilical artery, and that pre-treatment with the NO synthase inhibitor LNMA significantly attenuated the vasospasmic effect of ROS. The latter suggests that a component of the constrictor effects of ROS on the umbilical vascular bed may be mediated specifically by the inhibition of NO synthase activity in the endothelium (256).

Further data in the present manuscript show that, following I/R, both untreated and treated pregnancies responded with hypotension and sustained tachycardia. While the magnitude of the hypotensive effect was similar, the sustained tachycardia was significantly greater in allopurinol treated pregnancies. The dissociation between the magnitude and timing of the pressor and cardiac responses both suggest that baroreflex activation is unlikely to contribute to the mechanism increasing foetal heart rate. Rather, the enhanced tachycardic response in allopurinol treated pregnancies may again be due to an increase in NO bioavailability secondary to inhibition of ROS production, since NO is known to have a direct positive chronotropic effect. For instance, infusion of sodium nitroprusside in patients who had undergone a heart transplant increased heart rate prior to re-innervation of the heart (271). Further, administration of L-arginine in rats (272) and of sodium nitroprusside in guinea pigs (273), both led to sustained increases in heart rate in isolated heart preparations. Therefore, increased NO bioavailability in the foetal circulation in allopurinol treated pregnancies may have improved umbilical blood flow both by decreasing the constrictor effects of I/R-induced ROS production and by increasing cardiac output, secondary to an increase in foetal heart rate.

Interestingly, studies of heart failure in humans and in animal models have reported that XO activity is increased in the failing heart (274) and that allopurinol also possesses unique inotropic properties, increasing myocardial contractility while simultaneously reducing cardiac energy requirements (275-277). Whether these established cardioprotective effects of allopurinol extend onto the foetal heart is of obvious scientific and clinical interest, and warrants further investigation.

The mechanism mediating the fall in foetal arterial blood pressure following the I/R challenge is unclear. During umbilical cord compression, several investigators have reported that chemoreflex induced changes in sympathetic outflow promote increases in peripheral vascular resistance which contribute to the foetal hypertensive response (278;279). Following repeated compression of the umbilical cord, when chemoreflex activation ceases (279), foetal hypotension may result from the washout of lactate into the peripheral vascular beds (280). Accordingly, data in the present manuscript show that maternal treatment with allopurinol did not affect the magnitude of the foetal lactic acidemic response during repeated umbilical cord compression or the post-asphyxial hypotensive response.

In conclusion, this study tested the inter-related hypotheses that I/R-induced free radical generation will restrict umbilical blood flow secondary to activation of the xanthine oxidase pathway, and that maternal treatment with allopurinol will protect against this effect. The data presented strongly support the hypotheses tested and suggest that maternal treatment with allopurinol may offer plausible clinical intervention in the management of perinatal asphyxia in complicated labour.

Funding

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11

THE EFFECTS OF MATERNAL ALLOPURINOL ADMINISTRATION DURING REPEATED EPISODES OF ISCHEMIA-REPERFUSION ON FOETAL CARDIAC OXIDATIVE STRESS IN SHEEP

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Abstract

Background

The prevention and management of perinatal asphyxia remain major concerns in perinatal practice. Umbilical cord compressions (UCC) induce foetal asphyxia through episodes of ischemia-reperfusion (I/R). I/R promotes oxygen radicals, for instance via activation of the xanthine oxidase (XO) pathway, which may cause oxidative stress in the foetus. Allopurinol, a XO inhibitor, administered postnatally to severely asphyxiated human neonates was deemed too late to prevent oxidative damage. This study investigated the effects of maternal treatment with allopurinol on oxidative stress in the foetal heart following repeated UCC in late gestation sheep.

Methods

Five days after surgery, 10 foetal sheep instrumented with an inflatable occluder around the umbilical cord and an indwelling umbilical artery flow probe were submitted to an I/R challenge either during maternal allopurinol (n=5) or vehicle (n=5) infusion at 0.8 of gestation. Foetal hearts were collected 48h after I/R and snap-frozen for measurement of pro- and antioxidant proteins by Western blot. Hearts collected from 5 non-instrumented foetal sheep at 0.8 gestation served as age-matched controls.

Results

Episodes of I/R produced by repeated UCC led to increased expression of cyclooxygenase-2, endothelial nitric oxide synthase and heat shock protein 90, and decreased expression of manganese superoxide dismutase and gluthatione peroxidase in the foetal heart, findings consistent with cardiac oxidative stress. Maternal treatment with allopurinol ameliorated most of these effects.

Conclusion

The data show that I/R secondary to repeated UCC promotes oxidative stress in the foetal heart. Maternal treatment with allopurinol offers potential therapeutic treatment against this effect.

Introduction

During labour and delivery, foetuses commonly endure episodes of umbilical cord compression (UCC). Repeated compressions of the umbilical cord may induce foetal asphyxia and episodes of ischemia-reperfusion (I/R) (253). In complicated labour, perinatal asphyxia may result in hypoxic-ischemic encephalopathy, which is predictive of developing cerebral palsy and cognitive disability in later life (6). Therefore, the prevention and management of perinatal asphyxia remain major concerns in perinatal practice today.

It is known that I/R promotes the production of reactive oxygen species (ROS) (204). ROS can react with normal cellular compounds causing irreversible damage. Small amounts of ROS are commonly formed during normal metabolism and are scavenged by the body's natural antioxidant enzymes. However, during I/R the endogenous antioxidant capacity may become overwhelmed resulting in oxidative stress (204). One mechanism via which ROS are produced is through the activation of the xanthine oxidase (XO) pathway (257). Blocking this enzyme with a XO inhibitor, such as allopurinol, can reduce ROS formation (238). Therefore, the antioxidant capacity of allopurinol has gained increased attention in paediatric medicine and it is known that allopurinol can be safely administered to (preterm) neonates (76;77;238) and that it successfully reduces I/R-induced damage, for instance in paediatric cardiothoracic surgery (260). Results from early studies showed promising effects of treatment of asphyxiated newborns with allopurinol (76). However, contrary to expectation, postnatal allopurinol treatment was not effective in neonates with severe asphyxia, possibly because treatment was started too late (77). As a result, in pregnancy complicated by foetal asphyxia, recommendations to treat the foetus via the mother, rather than the neonate, are currently being entertained. Maternal oral administration of allopurinol during uncomplicated delivery has been shown to produce therapeutic levels of allopurinol in the neonate, even when the time between administration and birth was as short as 23 minutes (78), supporting that maternal treatment with allopurinol may offer a potentially interesting avenue for the prevention of perinatal asphyxia.

The data in chapter 10 of this thesis show that maternal treatment with allopurinol improved the recovery of umbilical blood flow and foetal survival following an I/R challenge produced by measured, repeated compressions of the umbilical cord in late gestation sheep possibly via maintenance of NO bio-availability due to reduced ROS production. The present study therefore investigated the effects of maternal treatment with allopurinol on pro- and antioxidant proteins in the foetal hearts.

Methods

The materials used in this study were collected from the animals subjected to the experimental protocol described in Chapter 10 of this thesis (see Chapter 10 for details of the surgical preparation, postoperative care and experimental protocol). Forty-eight hours after the end of the experimental protocol, ewes and foetuses were euthanised and the foetal hearts were collected and snap-frozen for measurement of pro- and antioxidant protein levels via Western Blot. Hearts collected from 5 non-instrumented foetal sheep at 0.8 gestation served as age-matched controls.

Western blotting

Tissue from the left ventricle was homogenised in ice-cold buffer (containing 40 mM Tris-Base, 2 mM EGTA, 1% Triton, 100 mM sodium pyrophosphate, 100 mM sodium orthovanadate, 1 M beta-glycerolphosphate, 1 M sodium fluoride and complete mini protease inhibitor cocktail (Roche Diagnostics, Manheim, Germany)) and centrifuged at 150,000 rpm. Aliquots were removed from the supernatant for protein quantification (BCA assay, Sigma, UK) and the supernatant diluted in a calculated volume of sample buffer (188.3 mM Tris Base, 416.1 mM SDS, 30% glycerol, 15% B-ME and 3% bromophenol blue) such that all supernatants were of equal protein concentration. Acrylamide gels varied from 7.5 to 10%, depending on the molecular weight of the protein of interest. Equal amounts of protein (20-30 ug) from each sample were loaded onto a 15-well gel after which proteins were separated by means of SDS-PAGE (sodium-dodecyl-sulphate-poly-acrylamide gel electrophoresis). Protein from the gel was transferred onto a nitro-cellulose membrane (pore size 0.2 uM; Invitrogen, Paisley, UK) via a semi-dry blotter. Once transfer was complete, membranes were stained with Ponceau to check for uniform protein loading and membranes were blocked at room temperature for 1 hour in blotto (5% dry milk in TBS-T) to decrease non-specific binding. Overnight incubation followed with specific primary antibodies at 4 degrees Celsius. After washing, membranes were incubated with a species-specific secondary antibody, followed by incubation with a substrate (ECL or ECL Plus kit, Amersham Biosciences, Bucks, UK) according to the manufacturer's instructions. The samples were then exposed to X-ray film. The levels of protein expression were quantified densitometrically and they were expressed as a ratio of protein loading.

Data and statistical analysis

All values for the expression of proteins in the Western analysis are expressed as percentage of control lysate for each experiment. Variables were assessed using either a

one-way or two-way ANOVA, as appropriate. Where a significant effect was indicated, the post hoc Tukey was used to isolate the statistical difference (Sigma-Stat; SPSS Inc., Chicago, IL, USA). For all comparisons, statistical significance was accepted when $P < 0.05$.

Results

Analysis by Western blot revealed a significant increase in the protein expression of cyclooxygenase-2 (COX-2), endothelial nitric oxide synthase (eNOS) and heat shock protein 90 (Hsp90) in hearts isolated from foetuses subjected to I/R during vehicle relative to hearts isolated from control foetuses (Fig. 1). Maternal treatment with allopurinol reverted the increase in foetal cardiac COX-2, eNOS but not Hsp90.

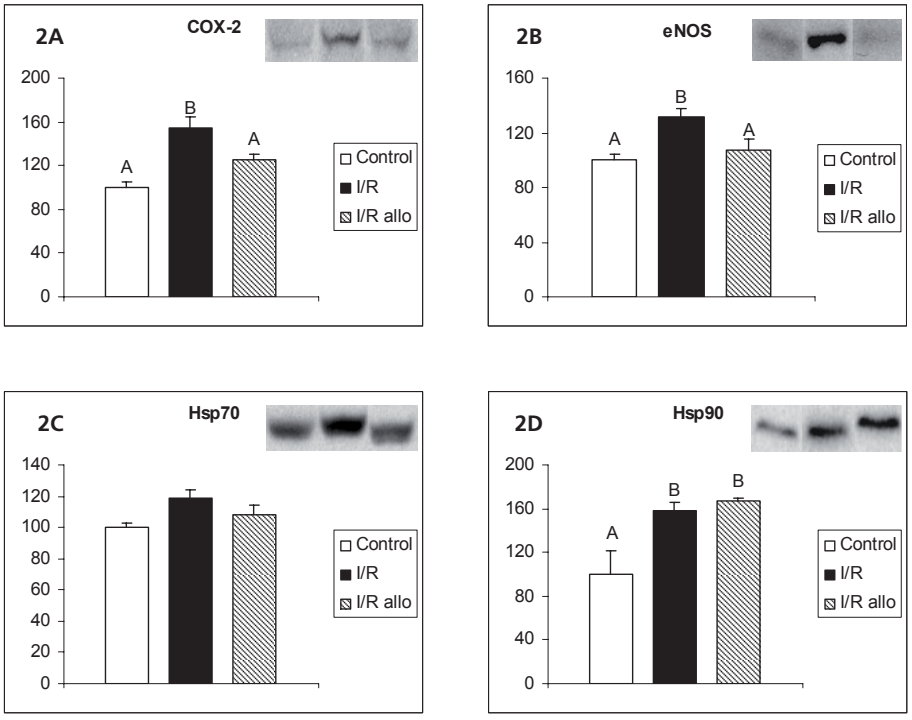

Figure 1 Oxidant proteins in the foetal heart
Data on the cardiac oxidant proteins are expressed as a percentage of control in 5 foetuses which received maternal i.v. treatment with allopurinol and 5 foetuses which received maternal i.v. treatment with buffered saline during the I/R challenge and 5 age-matched controls.
Different letters are significantly different $p<0.05$ (one-way ANOVA with Tukey test).

Similarly, levels of the antioxidant proteins manganese superoxide dismutase (mnSOD) and glutathione peroxidase (GPX) were significantly decreased in hearts isolated from foetuses subjected to I/R during vehicle relative to hearts isolated from control foetuses (Fig. 2). Maternal treatment with allopurinol partially reverted the decrease in foetal cardiac mnSOD and GPX. Although the changes in the protein expression of Hsp70 and catalase followed the same trends as for the other pro- and antioxidant proteins, respectively, these differences did not reach significance (Fig. 1 and 2).

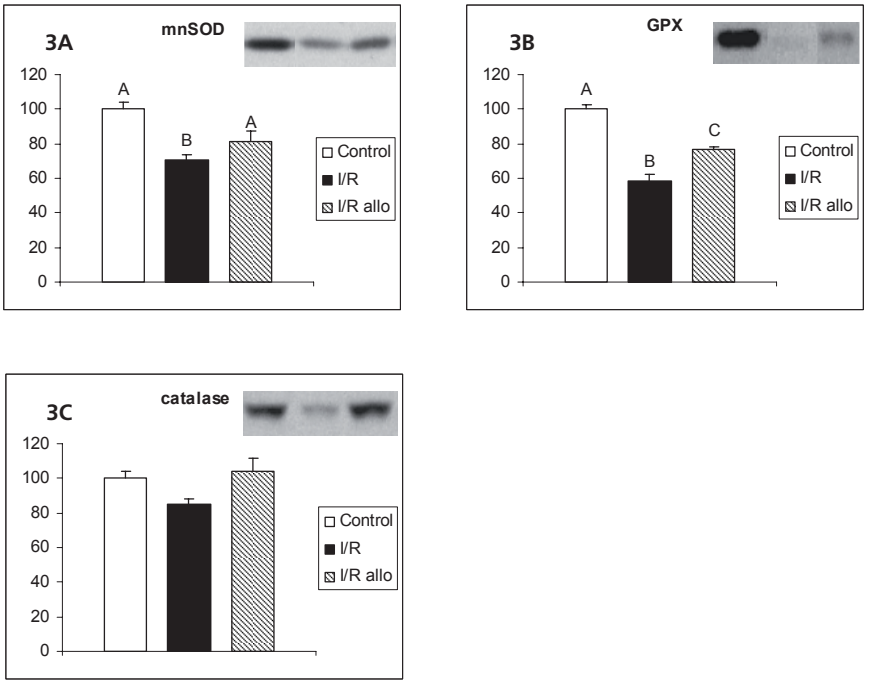

Figure 2 Antioxidant proteins in the foetal heart
Data on the cardiac antioxidant proteins are expressed as a percentage of control in 5 foetuses which received maternal i.v. treatment with allopurinol and 5 foetuses which received maternal i.v. treatment with buffered saline during the I/R challenge and 5 age-matched controls.
Different letters are significantly different $p<0.05$ (one-way ANOVA with Tukey test).

Discussion

These data show that episodes of ischemia-reperfusion produced by repeated compression of the umbilical cord led to increased expression of oxidant proteins and decreased expression of antioxidant proteins in the foetal heart, findings consistent with foetal cardiac oxidative stress. Maternal treatment with allopurinol ameliorated most of these effects.

The findings are consistent with activation of the xanthine oxidase pathway during I/R associated with repeated compression of the umbilical cord in complicated labour. The protective effects of allopurinol under such conditions may be explained by its pharmacological properties which not only directly prevent the formation of ROS by inhibition of the XO pathway, but also directly scavenge free radicals (239) as well as having the potential for non protein bound iron chelation (76;215).

In the present study, hearts isolated from foetuses subjected to I/R showed a significant increase in the protein expression of COX-2, eNOS and Hsp90 relative to control foetuses. COX-2 is induced in neonatal rat cardiomyocytes and hippocampi in response to oxidative stress (281;282). During ischemia, substantial amounts of arachadonic acid are released from membrane-bound lipids and following reperfusion COX-2 converts arachadonic acid and oxygen to prostaglandins and superoxide (283), thereby promoting further ROS production. The upregulation of COX-2 expression was prevented by maternal treatment with allopurinol, suggesting diminished ROS production via this route in pregnancies treated with maternal allopurinol. Xanthine oxidase (XO) and xanthine dehydrogenase (XDH) both catalyze the final two steps in purine metabolism and taken together XO and XDH are termed 'xanthine oxidoreductase' (XOR). XOR has been shown to be a regulator of COX-2 expression (284). These findings suggest that allopurinol prevents the induction of COX-2 through XO inhibition.

Endothelial nitric oxide synthase (eNOS) is a homodimeric oxidoreductase enzyme, which shuttles electrons between its reductase and oxidase domain in order to produce nitric oxide (NO). This gas is a potent vasodilator, signalling molecule, and inhibitor of leukocyte adhesion, platelet aggregation and cell proliferation (285). Although it is constitutively expressed, eNOS activity is regulated by a number of factors, including the heat shock protein 90 (Hsp90). The molecular chaperone Hsp90 can increase eNOS activity by increasing the rate of electron transfer between the two domains (286). In order to efficiently produce NO, eNOS requires L-arginine and O_2, as well as fully reduced tetrahydrobiopterin (BH4), which promotes the homodimerisation necessary for eNOS function (287). Without sufficient L-arginine, BH4 or O_2, eNOS becomes "uncoupled," producing H_2O_2 and $\cdot O_2$-. The data in this study show that episodes of I/R increase eNOS

and Hsp90 in the foetal heart, suggesting that episodes of umbilical cord compression in complicated labour may increase the susceptibility of eNOS to become uncoupled and produce further ROS. Interestingly, eNOS upregulation following I/R did not occur in foetuses whose mothers received allopurinol treatment. Decreased production of ROS secondary to activation of the xanthine oxidase pathway in allopurinol treated pregnancies may better maintain the bioavailability of NO, thereby preventing any need for eNOS upregulation. Further, excess NO is known to decrease eNOS expression, a negative feedback effect that has been demonstrated both *in vivo* and in the vascular tissues of rats following administration of a NO donor (288). This finding is also inkeeping with the better maintenance of blood flow in the highly NO-dependent umbilical circulation following I/R in the allopurinol treated pregnancies (see Chapter 10).

Manganese superoxide dismutase (mnSOD), catalase and glutathione peroxidase (GPX) are powerful endogenous antioxidant enzymes that contribute to the cell's ability to detoxify ROS. MnSOD converts the superoxide radical to hydrogen peroxide which, in turn, is detoxified by catalase and glutathione. Glutathione has been reported to play a far more important role as a hydrogen peroxide scavenger in the heart since its biological activity is much greater than that of catalase (289). Therefore, greater concentrations of glutathione may become consumed in the foetal heart following episodes of I/R, providing a suitable explanation for the significant depression of cardiac glutathione but not cardiac catalase in the foetus following umbilical cord compression. The diminished decrease in the expression of antioxidant enzymes in allopurinol treated pregnancies following I/R again suggests reduced ROS generation, alleviating the consumption of antioxidant defences in the foetal heart.

In conclusion, the data in this study show that episodes of I/R secondary to repeated compressions of the umbilical cord promotes oxidative stress in the foetal heart. Maternal treatment with allopurinol offers plausible clinical intervention for the reduction of foetal cardiac oxidative stress in complicated labour.

12

MATERNAL ALLOPURINOL TREATMENT DURING FOETAL HYPOXIA LOWERS CORD BLOOD LEVELS OF THE BRAIN INJURY MARKER PROTEIN S-100B

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Abstract

Background

Foetal hypoxia is an important determinant of neonatal encephalopathy due to birth asphyxia, in which hypoxia-induced free radical formation plays an important role.

Hypothesis

Maternal treatment with allopurinol (ALLO), a xanthine oxidase inhibitor and scavenger of free radicals, will cross the placenta during foetal hypoxia (primary outcome) and reduce S-100B and free radical formation (secondary outcome).

Methods

In a randomised double-blind feasibility study, 53 pregnant women in labour (54 foetuses) with a gestational age >36 weeks and foetal hypoxia (as indicated by abnormal/non-reassuring foetal heart rate tracing or foetal scalp pH<7.20) received ALLO 500 mg or placebo intravenously. Severity of foetal hypoxia and brain damage were assessed by arterial cord blood lactate and S-100B concentrations, respectively. Total hydroperoxide, thiol groups, isoprostanes and non protein-bound iron concentrations in cord blood were used to assess free radical formation. At birth, maternal and cord blood concentrations of ALLO and its active metabolite oxypurinol (OXY) were determined.

Results

ALLO and OXY concentrations were within the therapeutic range in the mother (ALLO >2mg/L and/or OXY >4mg/L), but not always in arterial cord blood. We therefore created 3 groups: a placebo (n=27), therapeutic ALLO (ALLO+; n=15) and subtherapeutic ALLO (ALLO-; n=12) group. Cord Lactate concentration did not differ, but S-100B was significantly lower in ALLO+ as compared to placebo/ALLO- groups (p<0.01). Less ALLO+ cord samples had measurable non protein-bound iron concentrations as compared to placebo (p<0.01).

Conclusions

Maternal ALLO/OXY crosses the placenta during foetal hypoxia though levels were not always in the therapeutic range. In foetuses/newborns with therapeutic ALLO concentrations lower plasma levels of the brain injury marker protein S-100B were detected. A larger ALLO trial in compromised foetuses at term seems warranted. The ALLO dosage must be adjusted to reassure therapeutic foetal ALLO/OXY concentrations.

Introduction

A more specific approach to improve neurodevelopmental outcome after perinatal hypoxia-ischemia or birth asphyxia remains an important issue. Moderate hypothermia has been proven to reduce brain damage after moderate-to-severe birth asphyxia (60;61;290;291). However, it is conceivable that combining this technique with a pharmacologic means of neuroprotection after birth asphyxia will further improve neurodevelopmental outcome (292). Several compounds have been shown to be promising candidates for this purpose in experimental and clinical studies (65;258;268;293). However, a major drawback in post-asphyxial hypothermia or pharmacologic treatment is the small therapeutic window (at best within 6 hours after birth) in which treatment should be initiated (292;294). This window is even smaller when one aims to prevent or reduce oxidative stress, an important cause of early reperfusion/reoxygenation injury to the immature brain. Free radicals are produced upon reperfusion and reoxygenation with maximal formation within the first 30 minutes after birth (19;295). Anti-oxidative treatment should therefore be started at birth or even before birth. Since foetal hypoxia is an important determinant in the aetiology of hypoxic-ischemic encephalopathy, maternal treatment with antioxidative drugs may be a more optimal approach for reduction of reduce reperfusion-reoxygenation injury after perinatal hypoxia-ischemia.
In the present feasibility study we investigated the effect of allopurinol (ALLO), a xanthine oxidase inhibitor and in higher concentrations also a direct scavenger of the toxic hydroxyl free radical and a chelator of non protein-bound iron (NPBI) (76;296;297) during foetal distress. Pregnant women with signs of foetal hypoxia, on the brink of delivery, were treated in a double blind randomised manner, with intravenously administered allopurinol or a placebo. There were several reasons for choosing ALLO: the increasing evidence that ALLO and its active metabolite oxypurinol (OXY) improve neurodevelopmental outcome after moderate-to-severe perinatal hypoxia-ischemia when used in the early neonatal period (77;268;298); its proven ability to cross the human placenta (78); and its proven safety profile (76-78;260;268). We hypothesised that maternally administered ALLO crosses the placenta during foetal hypoxia (primary outcome), reduces foetal free radical formation and ameliorates hypoxia-ischemia related brain damage on the short term as indicated by a decrease in foetal S-100B (secondary outcomes).

Methods

Women in labour (gestational age >36 weeks) with signs of foetal distress (as indicated by abnormal or non-reassuring foetal heart rate tracing or foetal scalp pH < 7.20) were treated in a double blind randomised manner with either a single intravenous dose of 500 mg ALLO or a placebo (saline). The dose of ALLO was derived from a study performed in healthy women in labour without foetal distress (78). Randomisation was performed by the Department of Pharmacy of the University Medical Centre Utrecht. Randomisation sequence was generated by a computer programme (Design, version 2.0 april 1998, Systat, Inc., Evanston, Illinois) in blocks of four. Patients were randomly assigned to treatment with allopurinol or placebo according to this randomisation sequence. Study drugs were prepared and labelled in a blind manner. Every site was supplied with study drug packages that were labelled with a unique set of numbers. The study drugs were stored at -20 degrees Celsius and defrosted on the ward immediately before use. Study medication was administered over 10 min after a thorough explanation and written consent from the mother and father, while the mother was being prepared for an emergency caesarean section or assisted vaginal delivery. In addition, women received acute tocolysis (either ritodrine or atosiban) in order to abolish further detrimental effects of contractions on the foetal condition. Women with signs of severe foetal distress were excluded from the study due to lack of time to properly prepare and perform the study protocol.

Maternal blood samples were obtained at the time of birth. Foetal blood samples were obtained from arterial cord blood. Maternal and cord blood investigations included measurement of plasma concentrations of ALLO and OXY and the free radical markers isoprostanes, total hydroperoxide, thiol groups and NPBI. Additionally, arterial cord lactate concentration was measured to assess the severity of foetal hypoxia (299). Protein S-100B concentration in arterial cord blood was used as a marker of central nervous system damage (300-302). Additionally, arterial cord plasma concentrations of liver functions (ALT; AST), renal function (urea, creatinine), uric acid and troponin-1 (myocardial function marker) were determined. Blood samples for ALLO and OXY determination were collected in heparinised tubes, centrifuged and stored at -20^0C until analysis. Blood samples for evaluation of oxidative stress were centrifuged at 2000 rpm for 10 minutes and the supernatant was stored with butylated hydroxytoluene, to prevent continuation of the oxidation process, at a temperature of -80^0 C.

All the neonates participating in the study were examined after birth. Adverse effects of ALLO on the white blood count and on the skin were monitored in mother and child (76;303).

The study was approved by the Scientific Boards and Ethical Committees of the participating hospitals (the University Medical Centre Utrecht, the University Medical Centre Groningen and the Universidad Del Norte in Barranquilla).

Analysis of ALLO, OXY, total hydroperoxide, thiol groups, isoprostanes and NPBI

ALLO and OXY plasma concentrations were determined using reversed-phase high-performance liquid chromatography with UV-detection at 254 nm for the quantification of ALLO and OXY in plasma (264). The method was linear between 0.5 and 25 mg/l with a lower limit of quantification of 0.2mg/l for both compounds. F2-isoprostanes in plasma were measured using the method of Morrow (304), by selected ion monitoring gas chromatography/negative ion chemical ionization-mass spectrometry employing [2H4] 8-iso-prostaglandin F2a as an internal standard. Total hydroperoxide production was measured with a d-ROMs Test (Diacron International, Italy). Thiol production was measured with –SHp Test (Diacron International, Italy), which is based on the ability of thiol groups to develop a photometrically detectable colored complex. The intensity of photometrically detected colour is directly proportional to the concentration of thiols. Finally, detection of NPBI in plasma is based on preferential chelation of NPBI by a large excess of nitrilotriacetic acid, low affinity ligand or NTA. NTA captures all iron bound to low molecular weight proteins and non specifically bound to serum proteins, however, it does not remove iron bound to transferrin or ferritin (305).

Statistical analysis

Data are summarised as means ± SD or as median and ranges (shown as Box-Whisker plots) where appropriate. Differences between 2 groups were compared by (unpaired) Student's *t*-test, Mann-Whitney U test or Chi-square test where appropriate. Differences between groups were assessed with one-factorial ANOVA followed by the Scheffe's procedure if a significant difference was found. A possible correlation was investigated with simple regression analysis. For statistical analysis STAT VIEW II (Abacus Concepts, Inc., Berkeley, CA) was used. Statistical significance was set at $p<0.05$.

Results

A total of 53 mothers and 54 infants were included in the present study: 27 placebo-treated mothers and 26 ALLO-treated mothers (1 multiple gestation). The ALLO-group was subdivided in a therapeutic (n=15) (ALLO+) and a subtherapeutic ALLO group (n=12)

(ALLO-) based on cord concentrations of ALLO and/or OXY (see also below). Table 1 summarises maternal characteristics as a function of treatment. No differences were found for any of the parameters shown.

Table 1 Maternal characteristics ([ALLO +] therapeutic allopurinol; [ALLO -] subtherapeutic allopurinol).

	ALLO+ (n=14)	ALLO- (n=12)	Placebo (n=27)
Nulliparity (n)	7	6	13
Maternal age (yrs) median [range]	30 [26-33]	30 [22-43]	33.5 [24-42]
Multiple gestation (n)	1	0	0
Time between infusion and delivery (min) median [range]	56 [24-190]	48 [18-94]	41 [12-449]
Instrumented vaginal delivery (n)	2	3	8
Caesarean section (n)	7	9	8

Data are presented as median [range] or (n).

Table 2 provides relevant perinatal data of the neonates in the various groups. Gestational age, birth weight, Apgar score, arterial umbilical cord pH and base excess did not differ between ALLO and placebo-treated newborns. Seventeen placebo, six ALLO- and five ALLO+ newborns had to be admitted to the neonatal ward; in respectively 8, 4 and 4 newborns this was related to preceding foetal hypoxia. Duration of admittance ranged from 3-to-16 days for placebo-treated infants (median stay: 7 days) and from 1-to-20 days for ALLO-treated infants (median stay: 8 days).

Table 2 Perinatal and neonatal data ([ALLO +] therapeutic allopurinol; [ALLO -] subtherapeutic allopurinol).

	ALLO+ (n=15)	ALLO- (n=12)	Placebo (n=27)
Gestational age (wks)	40 ± 1 4/7	40 1/7 ± 1 3/7	40 2/7 ± 1 5/7
Birth weight (grams)	3148 ± 351	3194 ± 740	3189 ± 584
Male sex (n)	8	8	15
Apgar score 5'	8.7 ± 1.3	9.3 ± 0.6	9.1 ± 0.8
Umbilical artery pH	7.17 ± 0.06	7.15 ± 0.02	7.14 ± 0.10
Umbilical artery BE	-10.3 ± 3.3	-8.3 ± 3.1	-8.9 ± 4.3

Data are presented as means ± SD or (n).

Table 3 summarises chemical markers with respect to important organ systems such as liver, heart and renal function. No differences were detected between ALLO and placebo-treated groups. Troponin was not elevated in any of the ALLO+ infants as compared to 2 and 4 infants in the ALLO- and placebo groups respectively.

Table 3 Important chemical markers from the arterial cord blood for liver, kidney , heart and brain ([ALLO +] therapeutic allopurinol; [ALLO -] subtherapeutic allopurinol).

	ALLO+ (n=15)	ALLO- (n=12)	Placebo (n=27)
AST (U/L)	43 [28-191]	40 [28-101]	44 [26-100]
ALT (U/L)	16 [7-21]	19 [17-26]	17 [7-42]
LD (U/L)	934 [541-5668]	1198 [439-4167]	1145 [352-1569]
Creatinine (umol/L)	73 [51-126]	68 [60-83]	67 [42-106]
Urea (mmol/L)	3.4 [2.7-6.7]	3.4 [1.4-4.5]	3.4 [1.8-7.3]
Uric acid (mmol/L)	0.36 [0.22-0.57]	0.30 [0.18-0.38]	0.32 [0.20-0.49]
Troponin (ug/L)	0 [0-0]	0 [0-0.12]	0 [0-0.28]

Data are presented as median [range].

Maternal and arterial cord ALLO and OXY concentrations at birth

The time from the start of maternal administration of ALLO or placebo to birth ranged from 18-to-190 (median: 56 min) and from 12-to-372 (median: 48 min) min respectively. Maternal ALLO and OXY concentrations were significantly higher as compared to arterial cord concentrations (Figure 1). Maternal ALLO and OXY concentrations ranged between 1.2 and 7.9 ug/ml (median: 3.8 ug/ml) and between 1.5 and 6.4 ug/ml (median: 3.2 ug/ml) respectively. The arterial cord ALLO and OXY concentrations ranged between 0.2 and 7.3 ug/ml (median; 2.0 ug/ml) and 0.6 and 7.6 ug/ml (median: 1.5 ug/ml) respectively. On basis of earlier (pharmacological) studies we considered an ALLO cord concentration of 2.0 ug/ml or more and OXY concentrations of 4.0 ug/ml or more in the therapeutic range in terms of xanthine oxidase inhibition (262;306). This was the case in 15 of the ALLO-treated newborns (ALLO+). The 12 remaining ALLO-treated newborns had subtherapeutic levels (ALLO-). OXY, but not ALLO, concentration in cord blood showed a positive correlation with time after maternal ALLO administration ($r=0.71$, $p<0.001$).

Arterial cord lactate and S-100B concentrations

Plasma lactate did not differ between groups (Figure 2). Plasma S-100B concentrations, however, were significantly lower in the ALLO+ group as compared to the placebo group (Figure 3A). Moreover, figure 3B shows a significant correlation between the sum of ALLO and OXY concentrations on the one hand and S-100B on the other hand ($r=0.59$, $p<0.001$).

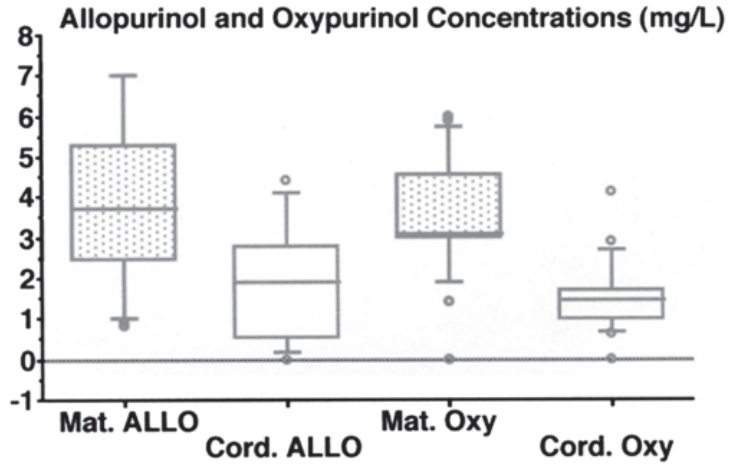

Figure 1 Maternal allopurinol (Mat. ALLO) and oxypurinol (Mat. OXY), and arterial cord allopurinol (Cord. ALLO) and oxypurinol (Cord. OXY) concentrations in mg/L at birth shown as Box-Whisker plots.

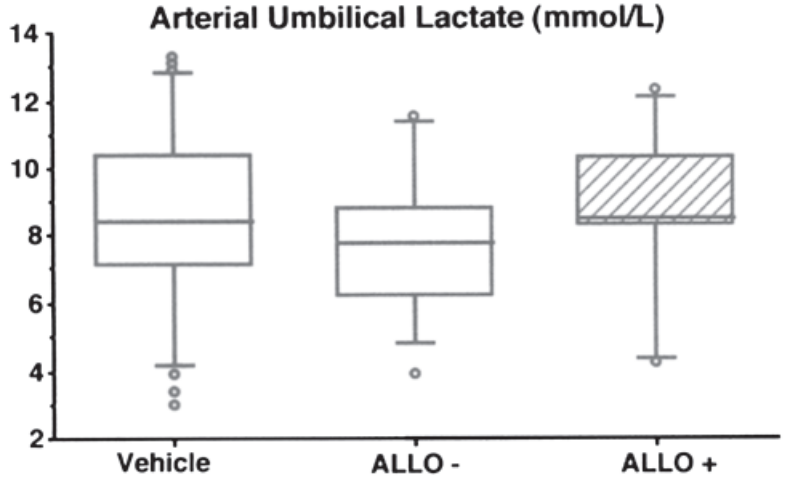

Figure 2 Arterial cord concentrations of lactate of the placebo-treated, the therapeutic allopurinol (ALLO +) and subtherapeutic allopurinol (ALLO -) groups shown as Box-Whisker plots.

Free radical markers in maternal and arterial cord plasma

Maternal free radical markers were always lower as compared to arterial umbilical samples, except for total hydroperoxide concentrations. No significant differences were detected between the 3 groups with regard to cord isoprostane, thiol groups, total

hydroperoxide or NPBI (Figure 4), although NPBI was present significantly more often in placebo and ALLO- cord blood as compared to ALLO+ cord blood (18/20, 10/10 and 7/15 respectively, $p<0.05$). Seven placebo and 2 ALLO- cord samples were haemolytic preventing reliable determination of NPBI.

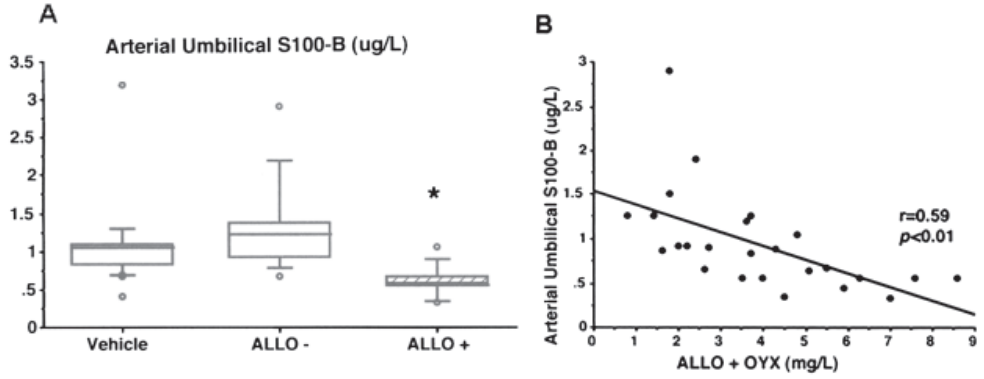

Figure 3 ***A.*** Arterial cord concentrations of S-100B of the placebo-treated, the therapeutic allopurinol (ALLO +) and subtherapeutic allopurinol (ALLO -) groups shown as Box-Whisker plots. * $p<0.01$ versus placebo/ALLO -.
B. Simple linear regression analysis between the sum of arterial cord concentrations of allopurinol and oxypurinol (ALLO+OXY) as a function of S-100B.

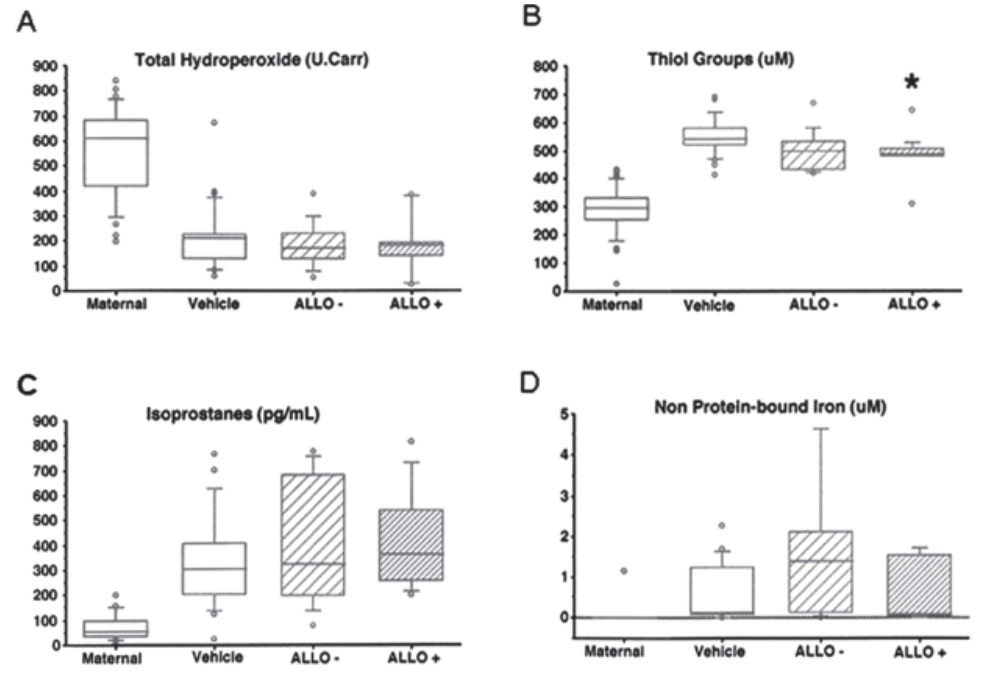

Figure 4 Maternal and arterial cord concentrations of the placebo-treated, therapeutic allopurinol (ALLO +) and subtherapeutic allopurinol (ALLO -) groups of total hydroperoxide, thiol groups, isoprostanes and non protein-bound iron shown as Box-Whisker plots.

Discussion

To our knowledge this pilot study is the first to show that ALLO administered to pregnant women in labour *with* foetal hypoxia crosses the placenta. Foetal ALLO concentrations, however, were somewhat lower in arterial cord blood in newborns who suffered from foetal hypoxia as compared to healthy newborns treated during uncomplicated delivery (78).

Furthermore, we found that, when therapeutic ALLO and/or OXY concentrations were present in the foetus, S-100B concentrations were substantially lower in arterial cord blood, suggesting less brain damage following foetal hypoxia.

However, several questions remain, which are partly due to the study set-up and the fact that the number of patients included in this pilot study was small. First, therapeutic ALLO and/or OXY levels were not reached in all neonates. This may have been due to the brief period between treatment and actual birth of the infant preventing optimal passage of ALLO from the mother to the foetus. This problem is difficult to solve since the obstetrician will prefer rapid delivery of the hypoxic foetus. Another reason for the sometimes subtherapeutical ALLO/OXY levels may be that the dosage used in this pilot study, which was based on placental passage of (orally administered) ALLO to healthy pregnant women in labour (78), was too low due to hindered placental passage as a result of suboptimal placental function or intermittent umbilical cord occlusion. Given the absence of any adverse effect of neonatal or maternal ALLO treatment in all human studies performed to date, a higher dosage should be considered (76-78;268). Although oral administration may be an option in labour complicated by foetal hypoxia, we could not detect ALLO or OXY in arterial cord blood of two additional cases with foetal hypoxia in which we treated the mother with 600mg of ALLO *orally* (unpublished observation).

Second, a short term chemical endpoint with respect to neurological outcome was evaluated in the present study; the S-100B concentration in cord blood. Although S-100B is accepted as a reliable estimate of brain damage in the newborn neonate (300-302), long-term follow-up with a detailed assessment of neurodevelopmental outcome of the children treated antenatally with ALLO or a placebo will be necessary to prove a neuroprotective effect of maternal ALLO treatment. We estimate that a total of 200-to-240 cases are needed for this type of study. This rather large number of inclusions is required because the outcome of the mature foetus suffering from foetal hypoxia is very variable as has been shown in earlier studies (reviewed in (3;307;308)).

A final comment should be made concerning the free radical marker concentrations measured in our study. No significant differences in free radical production were

detected between groups. This was not surprising for NPBI, since higher allopurinol concentrations are needed to significantly lower NPBI by chelation (297). In a way it is encouraging that we found lower NPBI concentrations (non-significant difference) and less cord blood samples in which NPBI could be detected in the therapeutic ALLO group. We have no clear explanation why the maternal total hydroperoxide concentrations were higher as compared to arterial cord levels, although the maternal range was quite large. The literature reports that total hydroperoxide levels are generally higher during delivery as compared to non-pregnant women (33). Furthermore, we are also unable to explain the lack of difference between groups with respect to plasma uric acid in the arterial cord blood. Assuming a substantial inhibition of xanthine oxidase activity in the therapeutic ALLO group, one would expect lower uric acid concentrations. We postulate that the time between ALLO administration and measurement of uric acid concentration may have been too short to assess differences in between groups. Finally, the small number of patients included in the present study may have masked possible differences in free radical marker levels and uric acid concentrations.

Despite the considerations and uncertainties mentioned above, the preliminary results including the significantly lower S-100B concentrations in the therapeutic ALLO group, make it worthwhile to design a larger trial. In this trial the most important end point should be a clear marker of long term neurodevelopmental outcome.

In summary, this pilot study shows that ALLO crosses the placenta during foetal hypoxia. Given the considerably lower umbilical cord ALLO and OXY concentrations in the present study as compared to concentrations measured after oral administration of an equivalent ALLO dose to mothers in uncomplicated labour (78), it is plausible that a higher ALLO dosage should be administered during foetal hypoxia to achieve therapeutic concentrations in the hypoxic foetus. This assumption seems realistic since all human studies to date in which ALLO was administered to the mother and foetus or neonate did not show any short or long term adverse effects. Furthermore, newborns with therapeutic ALLO concentrations in cord blood had lower S-100B concentrations. Therefore, a larger trial in compromised foetuses at term seems warranted.

Acknowledgements

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General discussion and summary

Pregnancies which become complicated by intrauterine growth restriction (IUGR), pre-eclampsia (PE) or haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome often have to be terminated prematurely. As a consequence of the early gestational age at delivery, foetuses born from these pregnancies are at increased risk of developing respiratory problems (309). It is unclear, however, whether placental insufficiency and maternal hypertensive disease have independent additional beneficial or detrimental effects on foetal lung maturation and clinical respiratory outcome in preterm pregnancy. This information may be important for obstetricians who have to decide on whether or not to administer antenatal steroid treatment and on the optimal timing of delivery.

Therefore, we tried to gain more insight into clinical respiratory outcome, foetal lung maturation and underlying pathophysiology of foetuses born from pregnancies complicated by IUGR and/or maternal hypertensive disease in the first part of this thesis (**Chapters 2 through 5**).

The **first aim** of this thesis was to determine whether respiratory outcome differs between preterm small for gestational age (SGA) foetuses with or without abnormal umbilical artery Doppler and/or maternal hypertension (**Chapter 2**). SGA foetuses with abnormal umbilical artery Doppler ultrasound examination are small due to placental insufficiency and are thought to represent the ' true' IUGR foetus. Previous groups (25-27) have aimed to study respiratory outcome of IUGR foetuses, however, they included wide gestational age (GA) ranges (25-42 weeks). This chapter reports the findings from our study in which strict inclusion criteria were used and correction for potential confounders was performed. Respiratory outcome of the 45 preterm SGA and 142 preterm IUGR foetuses included in our study was similar, indicating that placental insufficiency does not improve clinical respiratory outcome. In the relatively small study (n=61) by Sterne et al, no relationship between (degree of) Doppler abnormality and respiratory distress syndrome (RDS) was detected (27), whereas, contrary to our expectation, in two other publications a higher incidence of RDS was reported in the abnormal Doppler group (25;26). Unfortunately, in the large study by Soregaroli et al (26) no correction was performed for GA or birth weight, even though these variables varied considerably between Doppler-groups (GA 29 versus 34 and 37 weeks; birth weight 744 versus 1600 and 2120 grams in the reverse flow, abnormal pulsatility index (PI) and normal Doppler groups, respectively). Importantly, none of these studies included the maternal condition in the analysis.
The results from our study suggest that underlying maternal hypertensive disease may account for the different results. When we took maternal hypertensive disease into

account it became clear that IUGR foetuses from pregnancies complicated by HELLP syndrome had a significantly poorer respiratory outcome than IUGR foetuses born to mothers with normotension or PE. Two other research groups have also reported a poorer respiratory outcome in preterm infants born to mothers with HELLP syndrome (29;30).
One may suggest that respiratory outcome is poorer in infants from pregnancies complicated by HELLP syndrome due to an earlier GA at delivery and a lower incidence of antenatal steroid treatment in cases with severe HELLP syndrome. However, in the present study GA at delivery and rate of steroid administration were similar between PE and HELLP syndrome pregnancies (30.4±1.4 vs. 30.0±1.6 and 89% vs. 84%, respectively). Furthermore, the difference in respiratory outcome remained significant after controlling for these potential confounders. An explanation for the poorer respiratory outcome of IUGR foetuses born to mothers with HELLP syndrome could be that HELLP syndrome leads to increased oxidative stress in the IUGR foetus. Oxidative stress plays a role in RDS pathophysiology by causing lung tissue damage of the alveolar type II cell, inhibiting surfactant metabolism and causing surfactant inactivation (28), which may lead to detrimental neonatal respiratory outcome. Further research into the pathophysiological pathways leading to increased respiratory distress in neonates born to HELLP syndrome mothers and to quantify the extent of oxidative stress in the HELLP versus the PE foetus seems warranted.

Therefore, the **second aim** of this thesis was to study biochemical measures of foetal lung maturity in 76 SGA foetuses from pregnancies complicated by placental insufficiency (n=64) or maternal hypertension (n=55) (**Chapter 3**). The amniotic fluid lecithin to sphingomyelin (L/S) ratio is measured in our institute to determine foetal lung maturation when early delivery is expected. An L/S ratio > 2.0 has been shown to be an excellent predictor of favourable respiratory outcome (310). Unexpectedly, the results in this chapter showed that the L/S ratio of IUGR foetuses was significantly *higher* as compared to SGA foetuses. This indicates that foetal lung maturity is in fact enhanced in pregnancies complicated by placental insufficiency. These results differ from earlier clinical studies (25-27) and from the results reported in **Chapter 2** of this thesis. These studies were unable to confirm the hypothesis that placental insufficiency improves clinical respiratory outcome which may be explained by differences in study design. In the present chapter, a biochemical parameter of foetal lung maturation and not clinical respiratory outcome was studied. Biochemical parameters of lung maturation are measured objectively and on an absolute scale, whereas clinical respiratory outcome is measured subjectively and may be influenced by other (extrauterine) factors.

From recent clinical studies we had reason to believe that HELLP syndrome may cause respiratory morbidity (**Chapter 2** (29;30)). In line with these studies, this chapter reports significantly lower L/S ratios in pregnancies complicated by maternal hypertension. Subdivision of the maternal hypertension group showed that low L/S ratios were particularly found in pregnancies complicated by HELLP syndrome. Two previous studies also found that L/S ratios were lower in pregnancies complicated by maternal hypertension (104;105). In contrast, three other studies found no difference in L/S ratios between hypertensive and normotensive pregnancies (101-103). Importantly, none of those groups studied the effect of maternal hypertension in preterm SGA foetuses (GA <34 weeks, birth weight <p10 for GA). The present study shows that within the SGA population, HELLP syndrome in particular is associated with lower L/S ratios. Possibly, increased oxidative stress in mothers with HELLP syndrome leads to increased formation of free radicals in the foetus which may cause lung tissue damage or surfactant inactivation (28).

Thus, the **third aim** was to study oxidative stress and pro-inflammatory cytokine levels in 36 preterm neonates of pre-eclamptic mothers with or without HELLP syndrome to determine whether levels of oxidative stress were increased in HELLP syndrome as compared to PE (**Chapter 4**). RDS has been associated with oxidative stress and inflammatory processes (28;113-115), as have PE and HELLP syndrome (93;94;116;117) and oxidative stress levels have been shown to be correlated with maternal disease severity (94;109). This chapter confirmed our hypothesis that end-tidal carbon monoxide (ETCO: marker of oxidative stress in breath) and malondialdehyde (MDA: marker of lipid peroxidation in blood) levels are increased in infants born to mothers with HELLP syndrome as compared to PE. Pro-inflammatory cytokine levels were also increased at 0-12h hours. These results indicate that harmful pathophysiological pathways are (more intensely) activated in infants born to mothers with HELLP syndrome. These pathways may cause lung damage and surfactant inactivation (reviewed by (28)), providing a possible explanation for the increased RDS incidence (**Chapter 2**) and decreased L/S ratio (**Chapter 3**) found in these infants. It is unclear, however, how maternal oxidative stress causes foetal/neonatal oxidative stress. Placental passage, maternal placental interaction or placental/foetal responses to the maternal changes may be the effector mechanism. Further research to elucidate these mechanisms is required.

Therefore the **fourth aim** was to study placental histo(immuno)pathology in 164 placentae from IUGR pregnancies (**Chapter 5**). We aimed to study whether certain pathological features were specifically associated with PE or HELLP syndrome.

Placental infarction, which may occur due to defective maternal spiral artery transformation, was present in approximately 80% of cases (in line with (135;311)) and significantly more often in PE as compared to HELLP syndrome placentae which has recently also been reported by Vinnars et al (129). This is most likely due to earlier intervention due to worsening maternal clinical condition; management of PE is often expectant, providing more time for additional damage to occur in these already compromised placentae. This theory is supported by the fact that pregnancy was terminated for foetal reasons in 80% of PE as compared to 39% of HELLP syndrome pregnancies.

Early-onset PE has been associated with a pre-existent maternal pro-inflammatory phenotype and excessive production of pro-inflammatory cytokines and chemokines (reviewed by (143)). Interestingly, lymphocytic infiltration of foetal membranes was present significantly more frequent in PE and HELLP syndrome as compared to normotension which may reflect activation of the maternal immune system in response to the semi-allogenic foetus or an infectious agent.

Apoptotic syncytial knotting is part of normal placental development and remodelling (145) and occurs when degenerating apoptotic nuclei accumulate and protrude from the villous surface (146). This phenomenon ensures that placental cells that are no longer functional are eliminated without causing an inflammatory reaction in the mother (147). In our study, the presence of concomitant PE/HELLP syndrome was associated with increased knotting, indicating increased shedding of trophoblast. No difference in knotting was found *between* PE and HELLP syndrome placentae which is in line with Smulian et al (128). However, fascinatingly, HELLP syndrome placentae showed less apoptosis and more oxidative stress than PE placentae. We therefore speculate that increased oxidative stress causes a switch from apoptotic to necrotic trophoblast shedding in HELLP syndrome placentae, thereby reducing the rate of apoptosis relative to the PE placentae (150). Aponecrotic trophoblast causes an inflammatory response in the mother (147) which may provide an explanation for the more serious maternal condition of HELLP syndrome. The extent of placental necrosis in these placentae is currently being studied.

So, foetuses from pregnancies complicated by HELLP syndrome have more RDS (**Chapter 2**), a lower L/S ratio (**Chapter 3**), more neonatal oxidative stress (**Chapter 4**) and more placental oxidative stress (**Chapter 5**) as compared to foetuses from pregnancies complicated by PE.

Unfortunately, results regarding the respiratory outcome of preterm IUGR foetuses are less conclusive, with increased biochemical measures of foetal lung maturation (**Chapter 3**) but unchanged clinical respiratory outcome (**Chapter 2**) as compared to SGA foetuses. These findings imply that clinicians should not generally presume that IUGR foetuses will have a better respiratory outcome after early delivery. To enhance foetal lung maturation obstetricians often administer antenatal steroids which is according to general consensus that this treatment should be administered to women at risk of preterm birth (31-33). However, literature on antenatal steroid treatment in case of IUGR is scarce which might be due to the initial report by Liggins&Howie on possible adverse effects of steroids in pregnancies complicated by IUGR (34). Since then IUGR foetuses have been excluded from all large trials. Furthermore, in IUGR animal models steroids have been shown to cause brain damage, reduce brain growth and alter foetal cerebral blood flow (35-37).

The **fifth aim** was therefore to review the available literature on antenatal steroid treatment of infants with IUGR (**Chapter 6**). Because literature on the IUGR foetus specifically is sparse and many groups failed to distinguish between SGA and IUGR foetuses, we also decided to include reports studying the SGA foetus. Taken together, these observational studies suggest that antenatal steroids do not reduce neonatal morbidity or mortality (97) (**Chapter 2**, van Stralen (unpublished)) in the IUGR foetus. RDS incidence was not reduced in IUGR foetuses that had received antenatal steroids, possibly because lung maturation is already enhanced in these foetuses due to raised endogenous corticoid production associated with chronic intrauterine stress and breakdown of 11βHSD-II (the placental enzyme that converts maternal cortisol to its inactive metabolite cortisone) ((24;156) and increased L/S ratio (**Chapter 3**). Importantly, the incidence of intraventricular haemorrhage (IVH) was also similar between treated and untreated IUGR foetuses (97) (van Stralen (unpublished)). It is possible that IUGR itself not only enhances foetal lung maturation but also matures the central nervous system (169). This enhanced maturation may stabilise the endothelium of the germinal matrix causing the matrix to become less vulnerable for intracranial haemorrhage. At 2 years of age, a reduction in handicapped children has been reported in steroid treated IUGR foetuses; however, follow up at school age of this cohort did not show behavioural differences and unfortunately did not report on handicap free survival (97). At this age, physical growth below the 10th percentile was significantly more frequent in steroid treated infants. Finally, long term follow-up into adulthood has not been reported which is of importance because antenatal treatment is hypothesised to be a key mechanism underlying the foetal origins of adult disease hypothesis (158) as is IUGR

itself. Long term follow up of appropriately grown foetuses, however, failed to show any detrimental effects of a single course of antenatal steroids (165;166).
In the observational SGA studies, no difference in neonatal mortality between treated and untreated foetuses was reported. Results on neonatal morbidity in this population were inconclusive possibly due to heterogeneous study populations. None of the SGA studies reported whether cases had been evaluated for signs of placental insufficiency (as shown by abnormal umbilical blood flow Doppler studies and/or placental pathology). Furthermore, in 2 reports, type of steroid treatment was not strictly defined and assignment to the steroid treated group merely required that antenatal steroid treatment had been initiated and not necessarily completed (84;85) which may have resulted in treatment heterogeneity in the steroid group.
From the available literature, this chapter concluded that there is insufficient evidence that antenatal steroid treatment reduces neonatal morbidity and mortality in IUGR and SGA foetuses. On the basis of this chapter, we suggest that a randomised controlled trial should be performed to investigate whether treatment is beneficial in IUGR foetuses and to answer further questions regarding antenatal steroid treatment of SGA foetuses. The inclusion criteria of this trial should focus on the distinction between these two populations and the trial should evaluate both short and long term outcome.

Predictors for short and long term outcome are very important for the obstetrician who has to decide on the optimal timing of delivery of the IUGR foetus. Many monitoring techniques are therefore used in the management of IUGR (reviewed in (49-51)). Recently, several groups have suggested that magnetic resonance spectroscopy (MRS) could become available for non-invasive assessment of foetal wellbeing in IUGR (53-55) by measuring lactate concentration in amniotic fluid. However, to truly reflect the extent of foetal lacticemia, this concentration may have to be corrected for the amount of amniotic fluid (173) by calculation of the lactate to creatinine (L:C) ratio.
Therefore the **sixth** aim was to investigate whether amniotic fluid lactate concentration is a reliable parameter for estimating foetal lacticemia or if this concentration should be corrected for amniotic fluid volume (by determining the L:C ratio) (**Chapter 7**). To our knowledge this study is the first to report data on the correlation between simultaneously measured arterial umbilical cord lactate concentrations and amniotic fluid lactate and creatinine concentrations after caesarean section delivery of term (n=28) and preterm SGA (n=3) and IUGR (n=7) infants. The amniotic fluid L:C ratio has not yet been studied as a parameter of foetal wellbeing, however, in postnatal life, the urinary L:C ratio has been shown to be predictive of outcome in asphyxiated neonates (56).
A good deal of research into normal biochemical composition of the amniotic fluid was

performed many years ago and it has shown that metabolite concentrations change during normal pregnancy (176). With increasing gestational age, lactate concentration falls whereas creatinine has been shown to increase. This means that the L:C ratio can be expected to fall with increasing gestational age which was confirmed in this chapter. This chapter also showed that the L:C ratio is significantly correlated with arterial umbilical cord lacticemia, whereas amniotic fluid lactate concentration was not. This finding may be explained by the fact that the amniotic fluid lactate concentration is influenced by the amniotic fluid volume, similar to the lecithin concentration (174;175). Unfortunately, this pilot study was not designed to correlate foetal lacticemia results with neonatal outcome, which obviously is of great interest. Future studies should focus on measuring the amniotic fluid L:C ratio and on correlating this ratio with neonatal outcome. In future, when amniocentesis is performed to determine foetal lung maturity, the L:C ratio could be determined simultaneously (in merely 2 ml of AF) to provide more insight into the foetal condition. Furthermore, the L:C ratio may become important, as recent studies have demonstrated the capability to measure various amniotic fluid metabolites (including lecithin, lactate and creatinine) non-invasively via MRS or infra red spectroscopy (53-55;177). These non-invasive diagnostic tools could become valuable alternatives to invasive amniocentesis for the simultaneous assessment of foetal lung maturation and foetal acidosis.

Foetal acidosis was one of the predictors for outcome of the IUGR foetus that was studied in **Chapter 8**. Predictors for short term outcome have been studied extensively and these studies show that GA, birth weight and antenatal Doppler indices are all very important (26;39-43;45-48;312). Unfortunately, predictors for long term neurodevelopmental outcome and the placenta, a key organ dictating the intrauterine environment, were never assessed.
Thus, the **seventh aim** was to study neurodevelopmental outcome of 180 IUGR infants at 2 years of age and relate long term outcome to antenatal, perinatal and neonatal factors (**Chapter 8**). Long term outcome of the IUGR foetus was related to weight and acidosis at birth, indicating that the severity of IUGR and malnutrition affect long term outcome and that delivery may have to occur before foetal acidosis develops. Antenatal heart rate abnormalities have been shown to be related to hypoxemia (preceding foetal acidosis) (180;198) suggesting that abnormal foetal heart rate should be an indication for delivery. This is currently being studied in the TRUFFLE trial (*T*rial of *U*mbilical and *F*oetal *FL*ow in *E*urope) (199) in which computerised foetal heart rate variation and ductus venosus flow velocity waveform patterns are being studied as indicators of foetal impairment and delivery. Serial Doppler analysis may identify the cause of IUGR

(a defect in the placenta) rather than the consequence (foetal hypoxemia and acidosis) as reflected by an abnormal foetal heart rate pattern. Hopefully, the TRUFFLE trial will shed more light on the optimal timing of delivery of these compromised foetuses.
Placental villitis of unknown aetiology (VUE) was significantly associated with abnormal neurodevelopment which is in line with recent reports of long term neurological deficits in infants with placental VUE delivered at term (194;195). VUE was also associated with necrotizing enterocolitis (NEC) which has not been published previously, however, infants with NEC have been shown to have higher mortality rates and an increased risk for long term neurodevelopmental impairment (196). In the present study, NEC was not independently associated with mortality or neurodevelopmental outcome, indicating that confounding between VUE and NEC was not the case. The pathophysiological basis of the VUE associations found in this chapter need to be elucidated in future research. In the mean time, we recommend that placentae from pregnancies complicated by early-onset IUGR should routinely be examined for signs of VUE as presence of VUE may aid neonatologists in early identification of infants at increased risk of NEC, death and abnormal neurodevelopment.
Interestingly, neurodevelopmental outcome was not associated with IVH, periventricular leukomalacia (PVL), neurological examination at term or severe neonatal complications. Serious abnormalities on cranial ultrasound have been shown to be strongly associated with cerebral palsy and delayed mental development (183). The lack of association between IVH or PVL and neurodevelopmental outcome in the present study was possibly due to the fact that no infants had severe IVH or severe PVL. Resnick and colleagues have shown that adverse perinatal conditions lead to severe educational disabilities, but that less severe disabilities are more influenced by sociodemographic factors (200). Genetic factors, socioeconomical status and/or parental educational level may also have been of importance in determining long term outcome which is supported by the finding that variables significantly associated with neurodevelopment at 2 years of age accounted for only 22% of abnormal development in the present study. Unfortunately, we were unable to trace socioeconomical or parental educational level information for this cohort and due to small numbers no stratification into mild or severe developmental delay was possible.
Once again, antenatal steroid treatment was not significantly related to any of the neonatal outcomes (including RDS) in this group of IUGR foetuses which is in line with the findings presented in **Chapter 2** and the literature reviewed in **Chapter 6**.
Finally, this chapter confirmed previous findings that GA, birth weight and Doppler indices are important predictors for neonatal outcome in IUGR foetuses. Importantly, mortality decreased with increasing gestational age, but abnormal neurodevelopment

continued to be high, involving approximately 20% of infants born at the various ages. Previous studies have shown that GA is the most important variable determining short term outcome before 30 weeks of gestation. This chapter suggests that delivery of infants born after 30 weeks may have been too late in some cases, exposing them too long to continuing undernutrition.

So far, this thesis has focused on factors affecting outcome of the preterm IUGR foetus. As mentioned in the introduction, it would be of great clinical significance if an intrauterine therapeutic approach could be designed. Oxidative stress has been implicated in both IUGR and term asphyxia (11-17) and reducing oxidative stress may be beneficial in these foetuses. In the second part of this thesis we therefore focused on the first step towards developing an intrauterine approach for reduction of oxidative stress in term foetal asphyxia.

A great deal of research has been performed in term animal models of birth asphyxia. The **eight aim** of this thesis was therefore to review the available literature on therapeutic approaches for reduction of reactive oxygen species (ROS) after term asphyxia (**Chapter 9**). Many promising pharmacological means of neuroprotection (including selective iNOS and nNOS inhibition (62-66), erythropoietin (69-71), and allopurinol (17;73)) have been studied and since this review was written, early NFkappaB inhibition (67;68), xenon (74;75) and melatonin (72) have also emerged as promising interventions. Interestingly, when doing research for the review, hypothermia was emerging as a promising non-pharmacological approach in the treatment of term asphyxiated infants. In the mean time, head cooling for this purpose has actually been established in our neonatology ward, indicating that translational research is possible. Taking the complex pathophysiology into consideration, the most optimal intervention strategy for reducing ischemia reperfusion injury may be a combination of hypothermia and a pharmacological means of neuroprotection. At present, the most promising pharmacological compounds for translational research most likely are allopurinol and EPO, since these drugs have been administered to neonates without adverse side effects (76;77;231;238;260;268). Postnatal allopurinol treatment of the severely asphyxiated neonate, however, has been deemed too late to improve outcome (76;77). Allopurinol may be more effective when administered before reperfusion occurs which is possible through treatment of the foetus via the mother.

Therefore, the **ninth aim** was to study the effect of maternal allopurinol treatment on the foetal cardiovascular system in a late gestation sheep model of acute foetal asphyxia

(**Chapters 10 and 11**). The clinical situation of acute asphyxia during complicated labour was mimicked by repeatedly compressing the umbilical cord (reducing umbilical blood flow by 80-90% of basal values), leading to repetitive foetal heart rate decelerations and clinically relevant foetal acidemia. Six sheep received allopurinol during ischemia/reperfusion and six matched controls received a vehicle. First of all, **Chapter 10** shows that maternal allopurinol treatment during established foetal academia led to rapid increases in maternal and foetal plasma concentrations of allopurinol. In foetal plasma, elevations in allopurinol between 4 and 7 $mg.L^{-1}$ were achieved within 20 minutes of maternal administration, a range that is within the therapeutic levels described by Boda et al. (78). The fact that therapeutic levels of allopurinol were rapidly achieved in foetal plasma supports its use in the treatment of acute foetal asphyxia during labour**.**

Secondly, after the end of the experimental protocol allopurinol treated foetuses were able to restore umbilical blood flow to baseline values within 5 minutes following ischemia/reperfusion, whereas umbilical blood flow remained significantly depressed from basal values in untreated foetuses until 5 hours after the start of the challenge. These data suggest that ischemia/reperfusion increases the ratio of vascular superoxide to nitric oxide (NO) which worsens the effects on the foetus of asphyxia and acidosis by restricting umbilical blood flow to extents that may lead to foetal cardiovascular collapse and death. NO is important in preventing endothelial dysfunction and vasoconstriction (201). In the allopurinol treated foetuses, vasoconstriction in the umbilical artery may have been prevented by maintenance of NO bio-availability due to reduction of ROS.

Finally, **Chapter 10** shows that, following ischemia/reperfusion, both untreated and treated pregnancies responded with hypotension and sustained tachycardia. While the magnitude of the hypotensive effect was similar, the sustained tachycardia was significantly greater in allopurinol treated pregnancies. This is unlikely to be due to baroreceptor activation, but is also probably due to maintenance of NO bio-availability, secondary to inhibition of ROS production, since NO is known to have a direct chronotropic effect (271-273).

In conclusion, maternal allopurinol treatment has several effects on the foetal cardiovascular system which may be related to prevention of ROS. **Chapter 11** therefore reports on foetal cardiac oxidative stress parameters as measured by Western blot analysis.

COX-2 is induced in neonatal rat cardiomyocytes and hippocampi in response to oxidative stress (281;282). During ischemia, substantial amounts of arachadonic acid are released from membrane-bound lipids and following reperfusion COX-2 converts arachadonic acid and oxygen to prostaglandins and superoxide (283), thereby promoting further ROS production. The upregulation of COX-2 expression was prevented by maternal

treatment with allopurinol, suggesting diminished ROS production via this route in pregnancies treated with maternal allopurinol. 'Xanthine oxidoreductase' (consisting of XO and xanthine dehydrogenase) has been shown to be a regulator of COX-2 expression (284). These findings suggest that allopurinol prevents the induction of COX-2 through XO inhibition.

Endothelial nitric oxide synthase (eNOS) is an enzyme, which shuttles electrons between its reductase and oxidase domain in order to produce nitric oxide (NO). This gas is a potent vasodilator and although it is constitutively expressed, eNOS activity is regulated by a number of factors, including the heat shock protein 90 (Hsp90) (286). In order to efficiently produce NO, eNOS requires L-arginine and O_2, as well as fully reduced tetrahydrobiopterin (BH4) (287). Without sufficient L-arginine, BH4 or O_2, eNOS becomes "uncoupled," producing H_2O_2 and $\cdot O_2$-. The data in this study show that episodes of ischemia/reperfusion increase eNOS and Hsp90 in the foetal heart, suggesting that episodes of umbilical cord compression in complicated labour may increase the susceptibility of eNOS to become uncoupled and produce further reactive oxygen species. Interestingly, eNOS upregulation following ischemia-reperfusion did not occur in foetuses whose mothers received allopurinol treatment. Decreased production of ROS in allopurinol treated pregnancies may maintain the bioavailability of NO, thereby preventing any need for eNOS upregulation. Further, excess NO is known to decrease eNOS expression (288) which is also inkeeping with the maintenance of blood flow in the umbilical circulation in the allopurinol treated pregnancies (see **Chapter 10**).

Manganese superoxide dismutase (mnSOD), catalase and glutathione peroxidase (GPX) are powerful endogenous antioxidant enzymes that contribute to the cell's ability to detoxify ROS. MnSOD converts the superoxide radical to hydrogen peroxide which, in turn, is detoxified by catalase and glutathione. Glutathione has been reported to play a far more important role as a hydrogen peroxide scavenger in the heart since its biological activity is much greater than that of catalase (289). Therefore, greater concentrations of glutathione may become consumed in the foetal heart following episodes of ischemia-reperfusion, providing a suitable explanation for the significant depression of cardiac glutathione but not cardiac catalase in the foetus following umbilical cord compression. The diminished decrease in the expression of antioxidant enzymes in allopurinol treated pregnancies following ischemia/reperfusion again suggests reduced ROS generation, alleviating the consumption of antioxidant defences in the foetal heart.

In conclusion, **Chapter 11** shows that episodes of ischemia/reperfusion produced by repeated compression of the umbilical cord led to increased expression of oxidant proteins and decreased expression of antioxidant proteins in the foetal heart, findings consistent with foetal cardiac oxidative stress. Maternal treatment with allopurinol

ameliorated most of these effects.
Findings from **Chapter 10 and 11** suggest that maternal treatment with allopurinol offers plausible clinical intervention in the management of perinatal asphyxia in complicated labour.

Thus, the **final aim** of this thesis was to study maternal allopurinol treatment during term *human* labour complicated by foetal distress (**Chapter 12).** In this randomised double-blind feasibility study, 53 pregnant women in labour (54 foetuses) with a gestational age>36 weeks and signs of foetal hypoxia received allopurinol 500 mg (n=27) or placebo intravenously (n=27). In this chapter, we showed that maternal allopurinol crosses the human placenta during foetal distress during term labour. Unfortunately, allopurinol and oxypurinol levels were not always within the therapeutic range which may have been due to the brief period between maternal treatment and birth of the infant preventing optimal passage of allopurinol from the mother to the foetus. This problem is difficult to solve since the obstetrician will prefer rapid delivery of the hypoxic foetus. Another reason for subtherapeutical allopurinol levels may be that the dosage used in this pilot study, which was based on placental passage of *orally* administered allopurinol to healthy pregnant women in labour (78), was too low due to hindered placental passage as a result of suboptimal placental function or intermittent umbilical cord occlusion. Given the absence of any adverse effect of neonatal or maternal allopurinol treatment in all human studies performed to date, a higher dosage should be considered (76;77;238;260;268).
No differences were found in umbilical cord lactate concentration between groups, indicating a similar extent of foetal asphyxia. In infants with therapeutic levels, umbilical cord S-100B concentrations were significantly lower than those of placebo treated infants. Furthermore, a significant inverse correlation was present between allopurinol and S-100B concentrations, indicating a stronger prevention of brain damage with increasing allopurinol levels. Although S-100B is accepted as a reliable estimate of brain damage in the newborn neonate (300-302), long term follow-up with a detailed assessment of neurodevelopmental outcome will be necessary to prove a lasting neuroprotective effect of antenatal allopurinol treatment. We estimate that approximately 200-to-240 cases are needed to assess the effect of maternal allopurinol treatment on neurodevelopmental outcome after foetal hypoxia.
Finally, no significant differences in free radical production were detected. This was not surprising for non-protein bound iron (NPBI), since higher allopurinol concentrations are needed to significantly lower NPBI by chelation (297). In a way it is encouraging that we found less cord blood samples in which NPBI could be detected in the therapeutic

allopurinol group. Furthermore, we were unable to explain the lack of difference between groups with respect to plasma uric acid in the arterial cord blood. Assuming inhibition of xanthine oxidase in the therapeutic allopurinol group, one would expect to find lower uric acid concentrations. We postulated that the time between allopurinol administration and measurement of uric acid concentration may have been too short to assess differences in between groups. Finally, the small number of patients included in the present study may have masked possible differences in free radical marker levels and uric acid concentrations.

Despite the considerations and uncertainties mentioned above, these preliminary results make it worthwhile to design a larger trial in which the most important end point should be a clear marker of long term neurodevelopmental outcome. Funding for this trial has recently been obtained (ZonMW).

Summary

SGA and IUGR foetuses have a similar clinical respiratory outcome. Placental insufficiency does not seem to influence clinical respiratory outcome. Contrary to expectation, the L/S ratio is significantly higher in pregnancies complicated by placental insufficiency, indicating enhanced foetal lung maturation. This apparent contradiction may be due to differences in study design.

Maternal hypertension influences respiratory outcome; IUGR foetuses from pregnancies complicated by HELLP syndrome have a significantly poorer neonatal respiratory outcome than IUGR foetuses with otherwise healthy mothers. As expected, L/S ratios are significantly lower in pregnancies complicated by maternal hypertension. Low L/S ratios are particularly found in pregnancies complicated by HELLP syndrome.

Markers of oxidative stress and inflammation are increased in infants from mothers with HELLP syndrome as compared to PE. These results indicate that harmful pathophysiological pathways are (more intensely) activated in infants from mothers with HELLP syndrome. These pathways may cause lung damage and surfactant inactivation, providing a possible explanation for the increased RDS incidence in these infants.

HELLP syndrome is associated with more placental oxidative stress and less apoptosis as compared to PE. Increased oxidative stress may cause a switch from apoptotic to necrotic trophoblast shedding, thereby reducing the rate of apoptosis relative to PE. This is currently being studied.

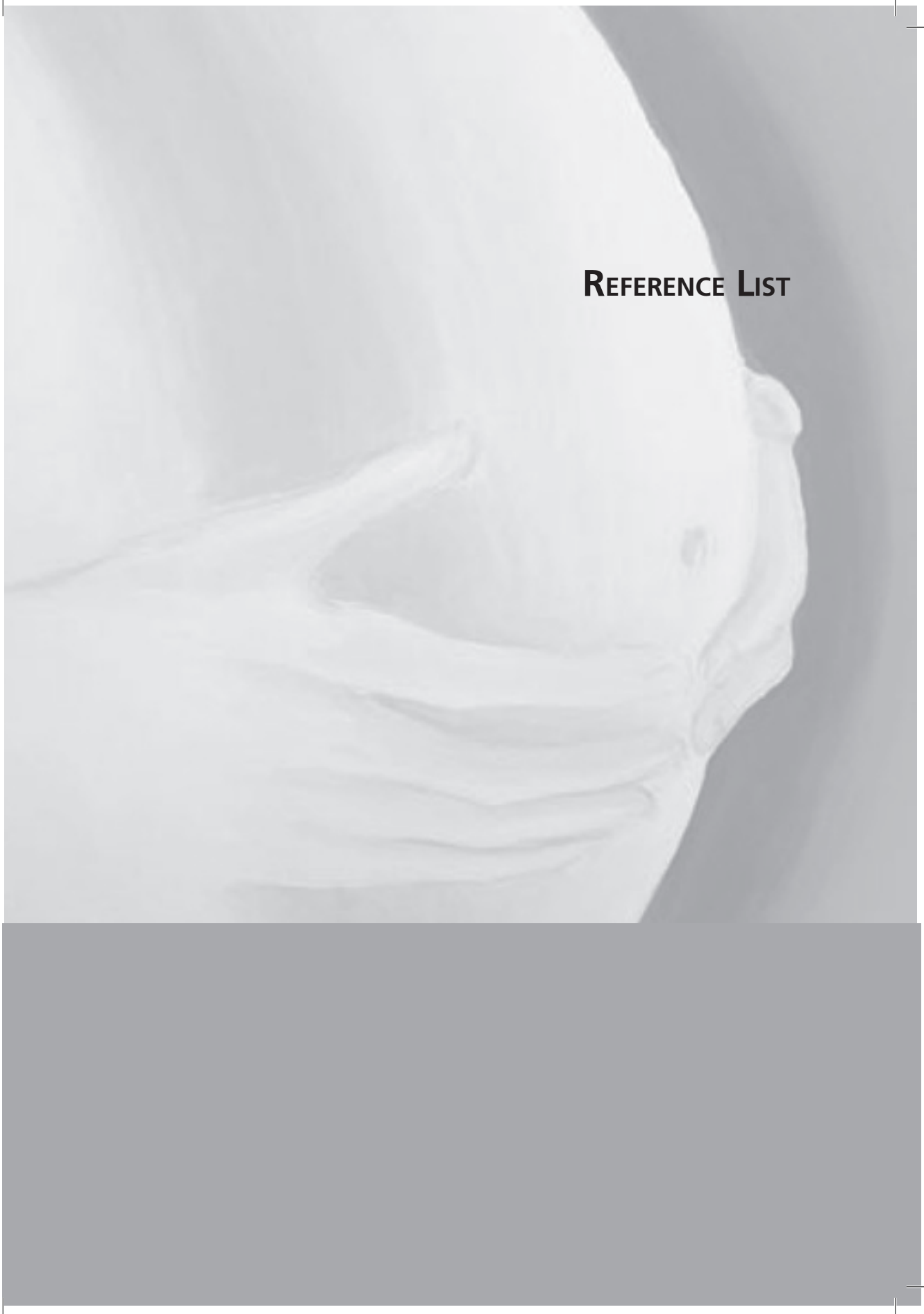
The amniotic fluid lactate:creatinine is a better predictor of foetal lacticemia than the amniotic fluid lactate concentration. When amniocentesis is performed to determine foetal lung maturation, the lactate:creatinine ratio could be determined simultaneously to provide more insight into the foetal condition. This ratio may be useful for research into new diagnostic tools for non-invasive measurement of metabolites in amniotic fluid.

There is insufficient evidence that antenatal steroid treatment reduces neonatal morbidity and mortality in IUGR foetuses. It remains unclear if antenatal steroid treatment is beneficial in SGA foetuses which may be due to heterogeneous study populations and treatment regimes. A randomised controlled trial should be performed to shed light on these matters.

The presence of placental villitis may aid neonatologists in early identification of preterm IUGR infants at increased risk of necrotizing enterocolitis, death and abnormal neurodevelopment. Abnormal neurodevelopment is also related to weight and acidosis at birth indicating that the severity of malnutrition and foetal acidosis affect long term outcome. Mortality decreases with increasing GA, but abnormal neurodevelopment continues to be high, involving approximately 20% of infants born at the various ages. GA has been shown to be the most important variable determining short term outcome before 30 weeks of gestation. Our study suggests that delivery of infants born after 30 weeks may be too late in some cases, exposing them too long to continuing undernutrition.

Maternal treatment with allopurinol reduces cardiac oxidative stress and improves the recovery of umbilical blood flow and foetal survival in a foetal sheep model of acute asphyxia.

In humans, maternal allopurinol crosses the placenta during foetal hypoxia although foetal levels are not always within the therapeutic range. In foetuses/newborns with therapeutic allopurinol concentrations, plasma levels of the brain injury marker protein S-100B are lower in cord blood. A larger trial in compromised foetuses at term seems warranted in which the allopurinol dosage must be adjusted and long term neurodevelopmental outcome should be studied.



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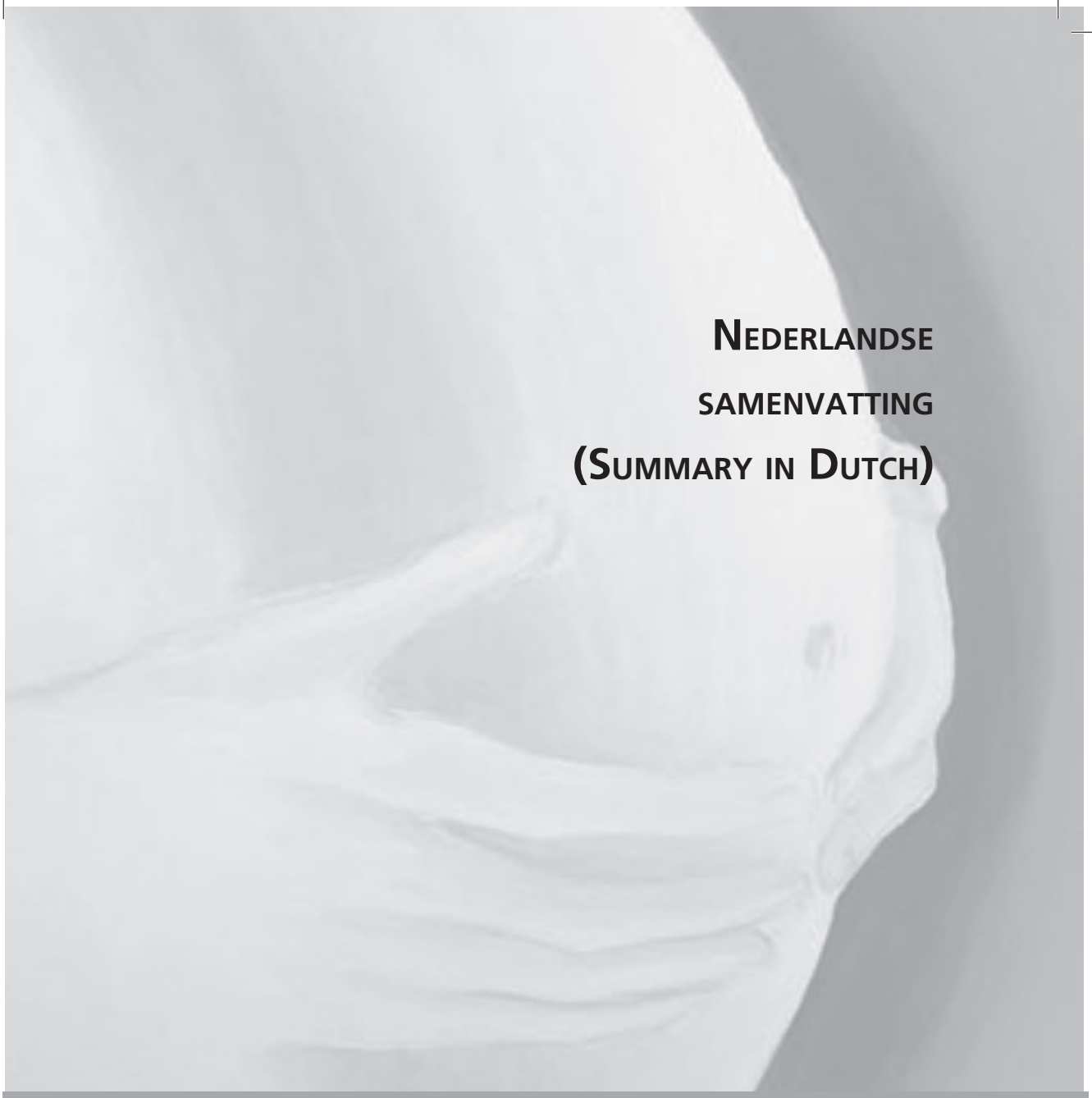


NEDERLANDSE SAMENVATTING (SUMMARY IN DUTCH)

Medische termen

Antenatale corticosteroïd behandeling: behandeling die wordt toegepast bij dreigende vroeggeboorte om de longen van de foetus sneller te laten uitrijpen. De behandeling wordt als compleet beschouwd indien twee keer een dosis betamethasone is geïnjecteerd in de bovenbeenspier van de moeder met een tijdsinterval van 24 uur en er minimaal 24 uur zijn verstreken na de laatste gift.
Apoptose: geprogrammeerde celdood
Asfyxie: zuurstofnood
A terme: een zwangerschapsduur van 37 weken of meer
Cerebrale parese: een blijvende beschadiging van de hersenen die zich voornamelijk uit in motorische en sensorische functiestoornissen.
Extra-uterien: buiten de baarmoeder
Foetus: het ongeboren kind vanaf de 12^{e} week van de zwangerschap tot aan de geboorte
Foetaal: de foetus betreffend
Fysiologie: functieleer van de organen
HELLP syndroom: ernstige moederlijke aandoening die kan optreden tijdens de zwangerschap en gepaard gaat met afbraak van rode bloedcellen, verhoogde leverenzymen en verlaagde bloedplaatjes
Intra-uterien: in de baarmoeder
Intra-uteriene groeirestrictie (IUGR): situatie waarbij de foetus vroeg in de zwangerschap in groei achterblijft door een verminderde werking van de placenta
Maternaal: de moeder betreffend
Mentale retardatie: ontwikkelingsstoornis met lager-dan-normale intelligentie en beperkte algemeen dagelijkse vaardigheden
Morbiditeit: ziekte
Mortaliteit: sterfte
Magnetic resonance spectroscopy (MRS): speciale vorm van MRI waarmee de aanwezigheid en hoeveelheid van bepaalde stoffen in het lichaam kan worden gemeten
Necrotiserende enterocolitis (NEC): zeer ernstige darmaandoening, waarbij de binnenbekleding van de darm afsterft, die zich bij vroeggeboren kinderen kan voordoen
Necrose: ongecontroleerde celdood
Neurologisch: het zenuwstelsel (hersenen, ruggenmerg en zenuwen) betreffend
Obstetricus: arts die zich specifiek bezighoudt met de verloskunde (zwangerschap en

geboorte)
Pathofysiologie: ziekteleer
Perinataal: de periode rondom de geboorte
Placenta: moederkoek
Preeclampsie (PE): 'zwangerschapsvergiftiging'; hierbij is er sprake van een te hoge bloeddruk en eiwituitscheiding in de urine.
Prematuur: te vroeg geboren (voor 37 weken zwangerschapsduur)
Respiratory distress syndrome (RDS): ademhalingsproblemen die kunnen ontstaan na vroeggeboorte door onvoldoende ontwikkeling van de foetale longen (ook wel hyaliene membraan ziekte genoemd)
'Small for gestational age' (SGA): pasgeborene met een geboortegewicht onder de 10^{e} gewichtspercentiel gecorrigeerd voor zwangerschapsduur en geslacht van het kind
Villitis: ontsteking van de placenta

Samenvatting in het Nederlands

Vroege intra-uteriene groeirestrictie (IUGR) en a terme asfyxie gaan beide gepaard met hoge perinatale mortaliteit en morbiditeit en soms met levenslange neurologische morbiditeit zoals cerebrale parese, leerstoornissen en mentale retardatie.

Hoewel de pathofysiologie van deze twee aandoeningen verschilt, is aangetoond dat oxidatieve stress in beide situaties een rol speelt. In het lichaam worden onder normale omstandigheden kleine hoeveelheden vrije radicalen gevormd. Vrije radicalen kunnen onherstelbare schade aanrichten door te reageren met bepaalde onderdelen van de cel, waaronder de celmembraan en het DNA. Daarom worden vrije radicalen gewoonlijk onschadelijk gemaakt door antioxidanten. Echter, wanneer vrije radicaalvorming sterk toeneemt, kan het antioxidatieve vermogen van het lichaam te kort schieten en ontstaat oxidatieve stress.
Het verminderen van oxidatieve stress zou positieve effecten kunnen hebben bij zwangerschappen die gecompliceerd worden door vroege IUGR of a terme asfyxie. In dit proefschrift wordt de invloed van oxidatieve stress op de foetale ontwikkeling bestudeerd (**deel 1**) en vervolgens wordt een eerste stap gemaakt in de ontwikkeling van een intra-uteriene behandeling ter vermindering van foetale oxidatieve stress (**deel 2**).

Deel 1 Oxidatieve stress en andere factoren die mogelijk invloed hebben op de prognose van de preterme IUGR foetus

Een abnormale aanleg en ontwikkeling van de placenta is geassocieerd met oxidatieve stress en speelt een belangrijke rol bij het ontstaan van zowel IUGR als maternale hypertensieve ziekten tijdens de zwangerschap (zoals preeclampsie (PE) of HELLP syndroom). Deze zwangerschappen worden vaak om maternale en/of foetale redenen vroegtijdig beëindigd. Doordat deze foetus bij een korte zwangerschapsduur worden geboren, lopen zij een groter risico op het krijgen van longproblemen ('respiratory distress syndrome' (RDS)). In het verleden is gesuggereerd dat de longen van de IUGR foetus versneld zouden uitrijpen door de langdurige intra-uteriene stress als gevolg van de abnormale placenta aanleg. Tot op heden is het echter nog onduidelijk of IUGR en maternale hypertensieve ziekte een *onafhankelijk* positief of negatief effect hebben op foetale longrijping en de klinische respiratoire uitkomst. Deze informatie is van belang voor obstetrici die moeten beslissen of zij antenatale corticosteroïden zullen toedienen om de foetale longen versneld te laten uitrijpen en moeten beslissen over het optimale tijdstip van geboorte van deze kinderen. Hierbij moeten de voordelen van verdere intra-uteriene rijping worden afgewogen tegen complicaties die kunnen optreden als gevolg van langdurige ondervoeding en zuurstofnood in de baarmoeder.

Daarom gaat **hoofdstuk 2** van dit proefschrift over de respiratoire uitkomst van 187 prematuur geboren 'small for gestational age' (SGA) kinderen, d.w.z. kinderen die te licht zijn voor de duur van de zwangerschap. SGA-kinderen van ouders die zelf klein van gestalte zijn, zijn fysiologisch SGA. Er zijn echter ook pathologische oorzaken voor het ontstaan van SGA, waaronder roken van de moeder, intra-uteriene infecties en een abnormale aanleg van de placenta. SGA-foetus met een afwijkende doorbloeding van de navelstrengarterie (wat gemeten kan worden met Doppler en echoscopie apparatuur) zijn klein voor de zwangerschapsduur door abnormale aanleg van de placenta en vormen daarom de IUGR foetus. In dit hoofdstuk wordt aangetoond dat RDS bij de prematuur geboren IUGR foetus even vaak voorkomt als bij de SGA foetus en dat maternale hypertensieve ziekte, vooral het zgn. HELLP syndroom, een negatieve invloed heeft op het functioneren van de longen direct na de geboorte.

In **hoofdstuk 3** wordt vervolgens de foetale longrijping van 76 prematuur geboren SGA foetus onderzocht. In de kliniek wordt de lecithine/sphingomyeline ratio in het vruchtwater (L/S ratio) gebruikt als maat voor foetale longrijping; een L/S ratio van meer dan 2 wordt beschouwd als een betrouwbare voorspeller voor een gunstige respiratoire uitkomst. In dit hoofdstuk wordt aangetoond dat de L/S ratio van de IUGR foetus hoger

is dan de L/S ratio van de SGA foetus, wat suggereert dat de longen van de IUGR foetus tóch sneller uitrijpen. Dit komt niet overeen met de resultaten in **hoofdstuk 2** van dit proefschrift, noch met eerder gepubliceerde studies. Deze discrepantie wordt mogelijk verklaard door verschil in studieopzet: de L/S ratio wordt objectief gemeten op een absolute schaal, terwijl klinische respiratoire uitkomst subjectief wordt bepaald en beïnvloed wordt door andere (extra-uteriene) factoren. Dit hoofdstuk bevestigt wel de bevindingen over HELLP syndroom uit **hoofdstuk 2:** in zwangerschappen gecompliceerd door het HELLP syndroom werden de laagste L/S ratio's gevonden.

In eerder onderzoek is aangetoond dat zowel RDS als PE en HELLP syndroom geassocieerd zijn met oxidatieve stress. Tevens is aangetoond dat de mate van maternale oxidatieve stress het hoogst is bij de meest zieke moeders. Het is daarom denkbaar dat toegenomen oxidatieve stress bij moeders met HELLP syndroom leidt tot sterkere vrije radicaalvorming (en uiteindelijke oxidatieve stress) in de foetus. Oxidatieve stress speelt een rol in de pathofysiologie van RDS omdat oxidatieve stress surfactant (een eiwit dat ervoor zorgt dat de longen zich na de geboorte goed kunnen ontplooien) onwerkzaam maakt en directe longschade veroorzaakt.

Daarom was het derde doel van dit proefschrift om oxidatieve stress te bestuderen bij 36 prematuur geboren IUGR kinderen van moeders met PE of HELLP syndroom (**hoofdstuk 4**). In dit hoofdstuk wordt bevestigd dat oxidatieve stress hoger is bij pasgeborenen van moeders met HELLP syndroom dan bij kinderen van vrouwen met PE. Dit suggereert dat schadelijke processen sterker geactiveerd zijn in kinderen van moeders met HELLP syndroom en dit vormt een mogelijke verklaring voor de slechtere respiratoire uitkomst (**hoofdstuk 2**) en verminderde longrijping (**hoofdstuk 3**) van deze kinderen. Het is echter onduidelijk hoe maternale oxidatieve stress leidt tot foetale oxidatieve stress; de placenta zou hierin een rol kunnen spelen.

Daarom hebben wij onderzoek gedaan naar 164 placenta's van deze foetus (hoofdstuk 5). Tijdens de normale ontwikkeling van de placenta vindt 'apoptotische knotting' plaats. Dit fenomeen zorgt ervoor dat niet-functionele placentacellen via apoptose worden afgestoten in de maternale bloedsomloop zónder een ontstekingsreactie te veroorzaken in de moeder. Uit dit onderzoek blijkt dat zowel PE als HELLP syndroom geassocieerd zijn met toegenomen knotting, wat suggereert dat afstoting van placentacellen in beide situaties is toegenomen. Hoewel er geen verschil in knotting werd aangetoond tussen PE en HELLP syndroom, was er wel minder apoptose en meer oxidatieve stress in placenta's van vrouwen met HELLP syndroom in vergelijking met PE . Op basis van

deze resultaten speculeren wij dat toegenomen oxidatieve stress in HELLP syndroom placenta's resulteert in een omschakeling van apoptotische naar necrotische afstoting van placentacellen. Necrotische afstoting van placentacellen zou kunnen leiden tot een ontstekingsreactie in de moeder en zou een verklaring kunnen vormen voor de ernstige maternale ziekte bij het HELLP syndroom. De mate van necrotische afstoting in deze placenta's wordt momenteel onderzocht.

Concluderend kunnen we stellen dat de IUGR foetus geboren uit een zwangerschap gecompliceerd door het HELLP syndroom meer RDS (**Hoofdstuk 2**), een lagere L/S ratio (**Hoofdstuk 3**) en meer neonatale en placentaire oxidatieve stress (**Hoofdstuk 4** + **5**) heeft dan de foetus geboren uit een zwangerschap gecompliceerd door PE.

Helaas zijn de onderzoeksresultaten over de premature IUGR foetus, bij wie geen sprake was van evidente moederlijke pathologie, minder eenduidig met enerzijds een betere foetale longrijping (**hoofdstuk 3**) maar anderzijds een onveranderd vóórkomen van RDS (**hoofdstuk 2**) vergeleken met de preterme SGA foetus. Clinici kunnen dus niet zomaar veronderstellen dat IUGR leidt tot een betere respiratoire uitkomst na vroegtijdige beëindiging van de zwangerschap. Volgens consensus dienen obstetrici vaak antenatale corticosteroïden toe bij dreigende vroeggeboorte om de foetale longen versneld te laten uitrijpen. Sinds de eerste studie door Liggins & Howie zijn echter de IUGR foetus altijd uitgesloten van deelname aan de grote gerandomiseerde onderzoeken. De reden hiervoor is dat in het onderzoek van Liggins & Howie werd aangetoond dat antenatale corticosteroïd behandeling leidde tot meer vruchtdood onder IUGR foetus. Inmiddels is ook uit experimenteel onderzoek gebleken dat antenatale corticosteroïden een nadelige invloed kunnen hebben in IUGR diermodellen (corticosteroïden veroorzaken hersenschade, verminderen hersengroei en veranderen de foetale bloedverdeling).

Het vijfde doel van dit proefschrift was dan ook om de beschikbare kennis over antenatale corticosteroïd behandeling van de IUGR foetus te bestuderen (**hoofdstuk 6**). Blijkens de beschikbare literatuur heeft antenatale corticosteroïd behandeling bij IUGR geen positief effect op neonatale morbiditeit (waaronder het vóórkomen van RDS) en mortaliteit . In slechts één studie is de uitkomst van corticosteroïd-behandelde versus corticosteroïd-onbehandelde kinderen op 2-jarige leeftijd onderzocht. Daarbij bleek een lagere incidentie van handicaps in corticosteroïd-behandelde kinderen. Op schoolgaande leeftijd werd er echter geen verschil gezien in gedrag. Helaas zijn handicaps op schoolgaande leeftijd niet meer bestudeerd en is er geen verder vervolgonderzoek verricht.

Op basis van deze bevindingen lijkt het ons reëel dat een gerandomiseerde studie wordt opgezet waarin onderzocht wordt of antenatale corticosteroïd behandeling in geval van IUGR zinvol is. In dit onderzoek moet duidelijk onderscheid worden gemaakt tussen IUGR en SGA foetus en dienen zowel de korte als lange termijn uitkomsten bestudeerd te worden.

Voorspellers voor korte en lange termijn uitkomst zijn zeer belangrijk voor obstetrici die het optimale tijdstip voor beëindiging van de zwangerschap gecompliceerd door IUGR moeten bepalen. Hiertoe worden verschillende technieken gebruikt, waaronder echografie en registratie van het foetaal hartritmepatroon. Recent is het idee ontstaan dat een speciale vorm van MRI (MRS: magnetic resonance spectroscopy) meer informatie over het welzijn van de foetus zou kunnen geven door het meten van lactaat (melkzuur) in het vruchtwater. Echter, bij foetale groeirestrictie is de hoeveelheid vruchtwater meestal afgenomen, waardoor de lactaatconcentratie verhoogd zou kunnen zijn (indikking), zonder dat sprake is van foetale nood. Doel van hoofdstuk 7 was dan ook om uit te zoeken of de vruchtwater lactaatconcentratie goed overeenkomt met de lactaatconcentratie in het foetale navelstrengbloed (en daarmee met de mate van foetale nood/acidose), of dat het lactaatgehalte in het vruchtwater gecorrigeerd moet worden voor de hoeveelheid vruchtwater door berekening van de lactaat:creatinine ratio (L:C ratio). In dit hoofdstuk wordt aangetoond dat de vruchtwater L:C ratio, maar niet de vruchtwater lactaatconcentratie, significant gecorreleerd is met het lactaatgehalte in het navelstrengbloed. Dit geeft aan dat de L:C ratio een betere voorspeller is voor foetale acidose en daarmee voor het foetale welzijn. Op dit moment zou in de kliniek, wanneer een vruchtwaterpunctie wordt verricht ter bepaling van de foetale longrijping, de L:C ratio mee bepaald kunnen worden als maat voor het foetale welbevinden. In de toekomst zal misschien gelijktijdige bepaling van foetale longrijping en foetale acidose via niet-invasieve MRS mogelijk worden.

Foetale acidose is in **hoofdstuk 8** van dit proefschrift bestudeerd als één van de voorspellers voor de uitkomst van de IUGR foetus. In het verleden zijn voorspellers voor de korte termijn uitkomst al uitgebreid bestudeerd, maar de placenta en de voorspellers voor de neurologische uitkomst op de langere termijn zijn niet eerder onderzocht. Daarom wordt in **hoofdstuk 8** van dit proefschrift de relatie bestudeerd tussen enerzijds antepartum en direct-neonatale factoren en anderzijds de ontwikkeling op 2-jarige leeftijd. De onderzoekspopulatie bestond uit 180 IUGR foetus die allen voor 34 weken zwangerschapsduur geboren werden. Zowel het geboortegewicht als foetale acidose bij de geboorte waren gerelateerd aan de ontwikkeling op 2-jarige leeftijd. Dit betekent

dat de ernst van de groeirestrictie en ondervoeding de lange termijn uitkomst negatief beïnvloedt. Daarnaast was villitis van onbekende origine in de placenta gerelateerd aan sterfte, necrotiserende enterocolitis (NEC) en abnormale ontwikkeling. Op basis van dit onderzoek adviseren wij dat IUGR placenta's routinematig onderzocht moeten worden op de aanwezigheid van villitis, omdat deze bevinding de neonatoloog zou kunnen helpen om 'at risk' neonaten vroegtijdig op te sporen.
In overeenstemming met de bevindingen in **Hoofdstuk 2** en **6** blijkt ook uit dit hoofdstuk dat antenatale corticosteroïd behandeling niet gerelateerd is aan de neonatale uitkomst van IUGR foetus. Verder wordt bevestigd dat zwangerschapsduur, geboortegewicht en Doppler onderzoek zeer belangrijk zijn voor de *korte* termijn uitkomst van de IUGR foetus. Een belangrijke nieuwe bevinding is dat de mortaliteit afnam met toenemende zwangerschapsduur, maar dat het vóórkomen van abnormale ontwikkeling op 2-jarige leeftijd hoog bleef. Uit eerdere studies is gebleken dat zwangerschapsduur de belangrijkste voorspeller is voor de korte termijn uitkomst van kinderen geboren vóór 30 weken. De bevindingen in deze studie suggereren dat de geboorte van kinderen na 30 weken in sommige gevallen te laat was, waardoor deze kinderen te lang bloot zijn gesteld aan ondervoeding en zuurstofgebrek.

Tot dusverre richt dit proefschrift zich op factoren die de uitkomst na vroege IUGR beïnvloeden. Voor de kliniek is het van groot belang om een intra-uteriene behandelingsstrategie te ontwikkelen. Oxidatieve stress speelt in de pathofysiologie van vroege groeirestrictie en a terme asfyxie een rol. In het tweede deel van dit proefschrift wordt daarom een eerste stap gemaakt in het ontwikkelen van een intra-uteriene behandelingsstrategie om foetale oxidatieve stress te verminderen.

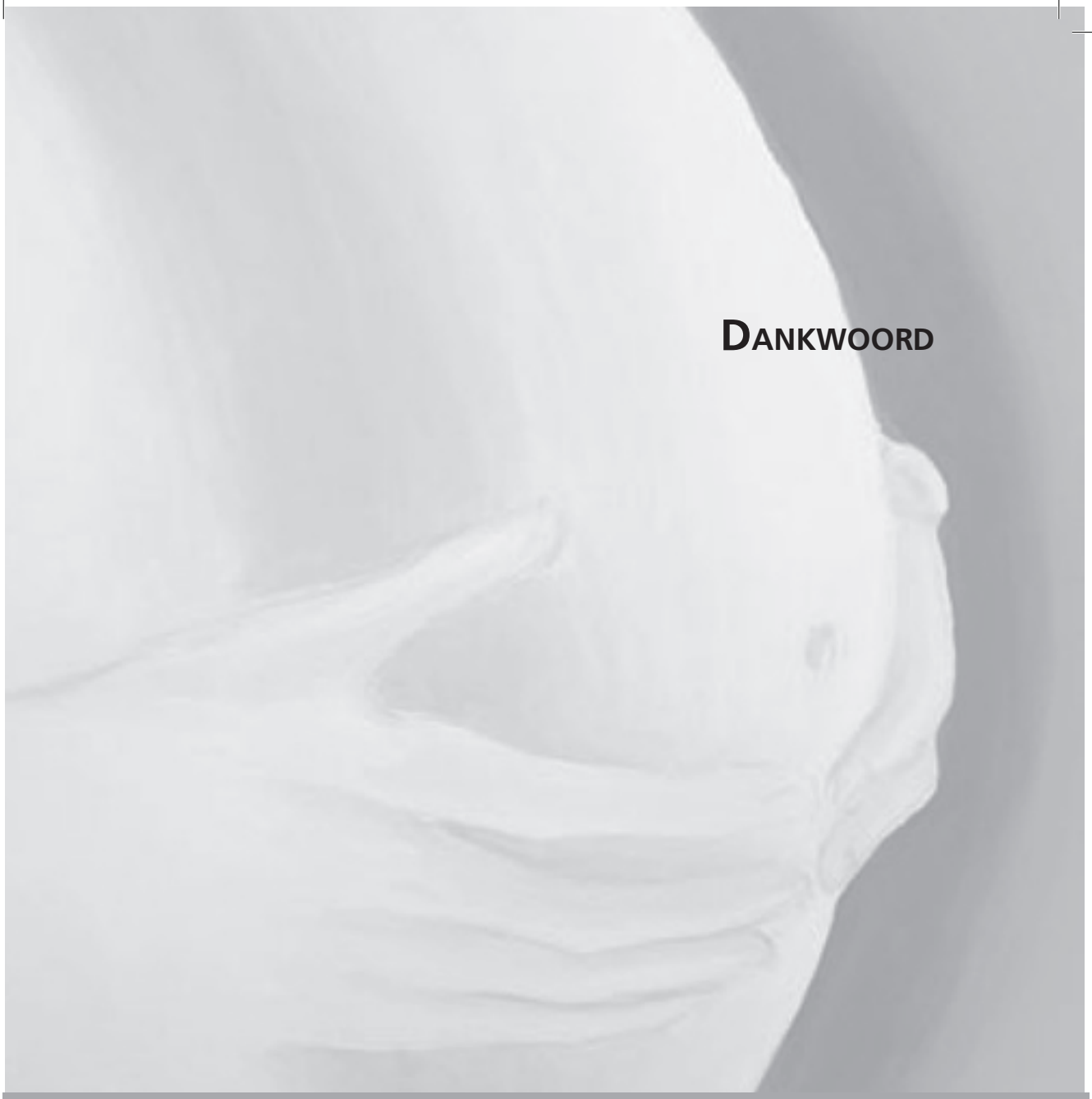
Deel 2 Behandelingsstrategie voor antenatale vermindering van foetale oxidatieve stress

In diermodellen van a terme asfyxie is al veel onderzoek verricht naar reductie van oxidatieve stress. In **hoofdstuk 9** wordt een overzicht gegeven van de bestaande literatuur. Tijdens het schrijven van dit hoofdstuk leek het koelen van de hersenen na asfyxie veelbelovend en in de tussentijd is deze behandeling op onze neonatologieafdeling geïnstitutionaliseerd. Door zuurstofnood worden echter meerdere schadelijke processen geactiveerd, waardoor de meest optimale behandelingsstrategie zou kunnen bestaan uit het combineren van koelen met medicatie, zoals allopurinol. Dit medicijn is bewezen effectief in het reduceren van vrije radicaalvorming. Helaas was postnatale behandeling van ernstig asfyctische neonaten met allopurinol niet zo effectief als gehoopt, mogelijk omdat de behandeling te laat gestart werd om schade te voorkomen. Allopurinol

zou wellicht effectiever kunnen zijn als het wordt toegediend op het moment dat zuurstofnood in de baarmoeder optreedt en het dus via de moeder toegediend wordt. Maternale allopurinol behandeling was echter tot op heden niet onderzocht, noch in dieren noch in mensen.
Daarom wordt in **hoofdstuk 10 en 11** maternale allopurinol behandeling in een foetaal schaapmodel bestudeerd. In deze studie is de navelstreng herhaaldelijk dichtgeknepen om de klinische situatie van zuurstofnood leidend tot acute asfyxie na te bootsen. In **hoofdstuk 10** wordt aangetoond dat maternale allopurinol behandeling zeer snel leidt tot therapeutische spiegels in het foetale schaap. Na het einde van het experimentele protocol bleef de navelstrengdoorbloeding in de allopurinol behandelde foetus op hetzelfde niveau als dat van voor de start van het protocol, terwijl dit in de onbehandelde foetus niet het geval was. Ook trad minder sterfte op in de groep die behandeld was met allopurinol. Een verklaring zou kunnen zijn dat allopurinol ervoor zorgt dat stikstofoxide (NO) beschikbaar blijft in het lichaam door vermindering van vrije radicaalvorming: NO is zeer belangrijk in het voorkómen van het samenknijpen van de bloedvaten. In **hoofdstuk 11** wordt aangetoond dat ernstige asfyxie leidt tot verhoogde aanwezigheid van oxidatieve eiwitten en verlaagde aanwezigheid van antioxidatieve eiwitten in het foetale schapenhart, passend bij het ontstaan van oxidatieve stress. Maternale allopurinol behandeling verminderde de mate van oxidatieve stress in het foetale schapenhart. Samengevat suggereren de bevindingen in **hoofdstuk 10 en 11** dat maternale allopurinol behandeling mogelijk een veelbelovende strategie is voor de behandeling van zuurstofnood tijdens de bevalling.

Daarom wordt in het **laatste hoofdstuk** van dit proefschrift maternale allopurinol behandeling tijdens foetale nood bij de mens onderzocht. In een prospectief onderzoek tijdens de bevalling werden 53 vrouwen bij wie er aanwijzingen waren voor foetale nood gerandomiseerd en kregen allopurinol of een placebo toegediend. In dit onderzoek werd ook de placentapassage van allopurinol tijdens foetale nood bepaald. Helaas waren de spiegels in het navelstrengbloed niet altijd therapeutisch, wat mogelijk wordt verklaard door de korte tijd tussen maternale toediening en de geboorte van het kind. Dit probleem is moeilijk op te lossen aangezien de obstetricus een snelle geboorte van het kind zal verkiezen indien sprake is van foetale nood. De soms subtherapeutische spiegels zijn mogelijk ook te wijten aan het feit dat de dosis te laag was. De dosis was gebaseerd op placentapassage in een eerder uitgevoerde studie waarin allopurinol oraal werd toegediend aan vrouwen tijdens een ongecompliceerde bevalling. Placentapassage tijdens foetale nood zou suboptimaal kunnen zijn door verminderde placentafunctie of door herhaaldelijke compressie van de navelstreng. Wij overwegen daarom om de

dosis te verhogen, mede gezien het feit dat tot op heden in geen van de neonatale of maternale studies negatieve effecten van allopurinol zijn waargenomen.
Bij pasgeborenen met een therapeutische allopurinol spiegel werden significant lagere waarden van een marker voor hersenschade, het S-100B, gevonden. In de toekomst zal een grotere (inmiddels door ZonMW gefinancierde) trial moeten uitwijzen of maternale allopurinol behandeling tijdens foetale nood inderdaad tot een betere neurologische uitkomst op korte en lange termijn leidt.



Dankwoord

Dankwoord

Als u op deze pagina bent gekomen na alle pagina's gelezen te hebben dan zult u snappen dat dit proefschrift niet tot stand was gekomen zonder medewerking van veel personen. Graag wil ik hieronder een aantal mensen in het bijzonder bedanken.

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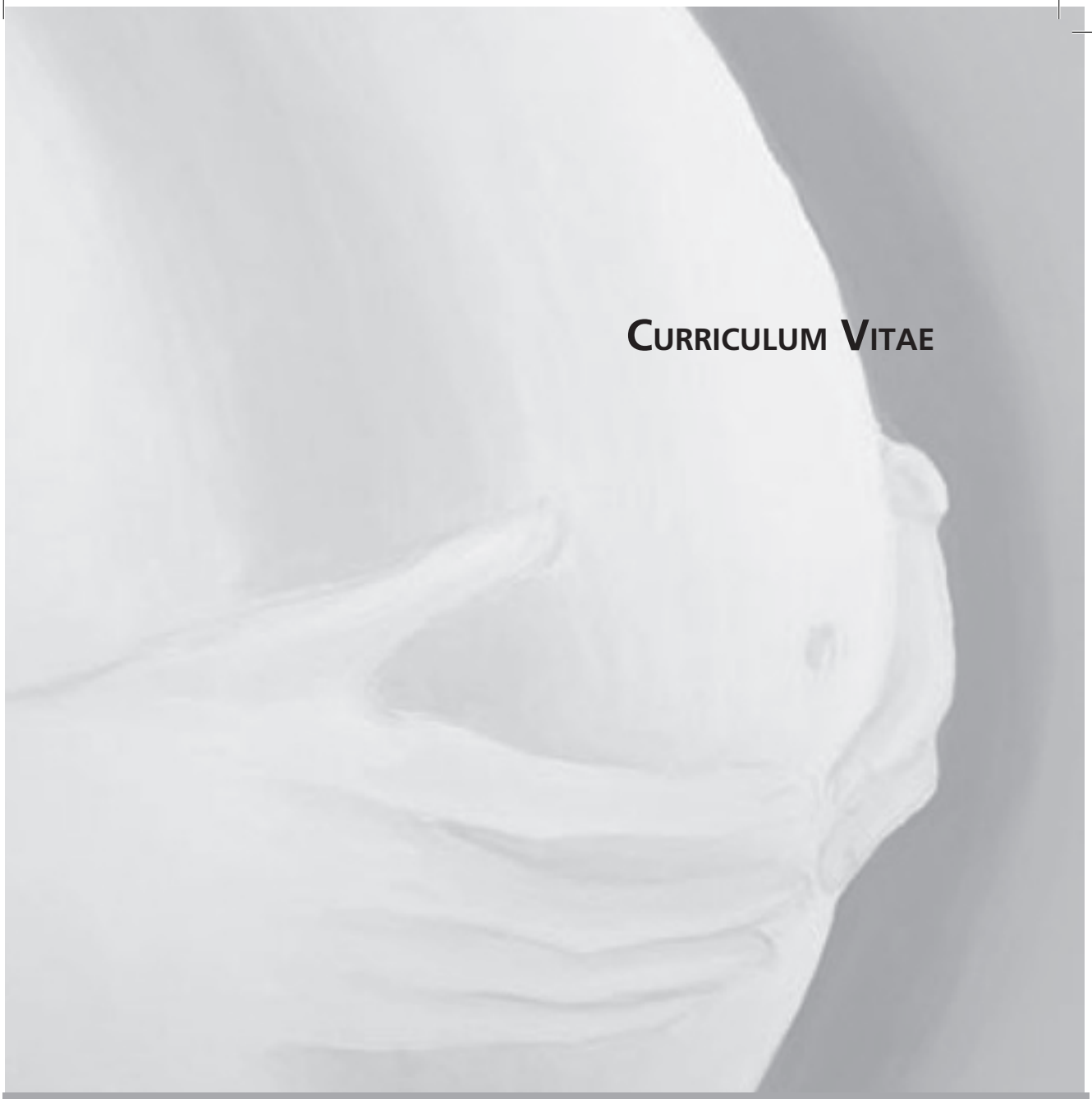
Allerliefste Sytse, laten we nog lang samen genieten!



Curriculum Vitae

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Helen Laura Torrance werd op 29 maart 1981 te Leiden geboren als zesde dochter van Schotse ouders. In 1999 behaalde zij het V.W.O. diploma aan het Thomas More College te Oudenbosch waarna zij in Utrecht aan de studie Geneeskunde begon. Zij startte in 2005 na haar afstuderen aan een promotietraject onder leiding van Prof. dr. G.H.A. Visser en Prof. dr. F. van Bel. Tijdens dit traject heeft zij enkele maanden onderzoek verricht aan de University of Cambridge en enkele maanden als ANIOS Obstetrie gewerkt in het Wilhelmina Kinderziekenhuis te Utrecht. Op 15 augustus 2008 is zij begonnen aan haar opleiding tot gynaecoloog in het TweeSteden ziekenhuis te Tilburg (opleider dr. H.J.H.M. van Dessel).