Chapter 2

Low plasma concentrations of arginine and asymmetric dimethylarginine in premature infants with Necrotizing Enterocolitis (NEC)

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Abstract

Introduction
Several studies have described reduced plasma concentrations of arginine, the substrate for nitric oxide synthase (NOS) in infants with necrotizing enterocolitis (NEC). No information on the plasma concentrations of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) in patients with NEC is currently available. We investigated whether plasma concentrations of arginine, ADMA, and their ratio differ between premature infants with and without NEC, and between survivors and non-survivors within the NEC group.

Methods
In a prospective case–control study, arginine and ADMA concentrations were measured in ten premature infants with NEC (median gestational age 193 d, birth weight 968 g), and ten matched control infants (median gestational age 201 d, birth weight 1102 g), who were admitted to the Neonatal Intensive Care Unit.

Results
In the premature infants with NEC, median arginine and ADMA concentrations (mmol/l), and the arginine/ADMA ratio were lower compared to the infants without NEC: 21.4 v. 55.9, P=0.001; 0.59 v. 0.85, P=0.009 and 36.6 v. 72.3, P=0.023 respectively. In the NEC group, median arginine (mmol/l) and the arginine/ADMA ratio were lower in non-surviving infants than in surviving infants: 14.7 v. 33.8, P=0.01 and 32.0 v. 47.5, P=0.038 respectively. In premature infants with NEC not only the NOS substrate arginine, but also the endogenous NOS inhibitor ADMA and the arginine/ADMA ratio were lower than in infants without NEC. In addition, low arginine and arginine/ADMA were associated with mortality in infants with NEC.

Conclusions
Overall, these data suggest that a diminished nitric oxide production may be involved in the pathophysiology of NEC, but this needs further investigation.

Introduction

Necrotizing enterocolitis (NEC), is the most common gastrointestinal emergency in the premature infant (1). Although NEC is a multifactorial disease, prematurity is a main risk factor. Mucosal injury resulting from ischemia, bacterial colonization, and early enteral feeding are also recognized as potentially important contributors to the pathogenesis of NEC (2;3).

It has been shown that in premature infants at the time of the diagnosis NEC, arginine plasma levels are decreased (4;5). Arginine is one of the most versatile amino acids in mammalian cells, which is crucial for ammonia detoxification and serves as the precursor of nitric oxide (NO) (6;7). NO plays an important role in several pathophysiological aspects of critical illness such as infection, organ injury, but also in inducing gut smooth muscle relaxation, the regulation of mucosal blood flow and maintaining mucosal integrity and the intestinal barrier function (8).

In an experimental piglet model, it was shown that arginine could have therapeutic potential in NEC by attenuating intestinal injury, whereas administration of the NO synthase inhibitor L-NAME caused haemorrhagic congestion of the gut wall (9). Previously, we showed in a rat model that low arginine plasma levels in combination with a low dose of endotoxin compromised blood flow through the small intestine (10).

Interestingly, Amin et al. (11), showed that arginine supplementation in premature infants, reduced the incidence of all stages of NEC.

Recent insights into NO metabolism have shown an important role of endogenously produced inhibitors of NO synthase, in particular asymmetric dimethylarginine (ADMA) (12). ADMA as well as symmetric dimethylarginine (SDMA), are synthesized when arginine residues in proteins are methylated by the action of protein arginine methyltransferases (PRMT). ADMA is an endogenous inhibitor of all isoforms of NO synthase (NOS), while SDMA is not.

Arginine is transported into endothelial cells by the cationic amino acid transporters (CAT) of system y^+, where it serves as a substrate for NO synthesis (13). Thus, the arginine/ADMA ratio is an important determinant of NO production by NOS (14;15).

Elevated plasma levels of ADMA have been found in diseases related to endothelial dysfunction including peripheral arterial disease, hypertension, hyperlipidemia, diabetes mellitus and hyperhomocysteinemia (16). Interestingly, ADMA have been shown to independently predict intima media thickness of the carotid artery in individuals without clinical manifestations of arteriosclerosis (17;18). Furthermore, it has been shown that ADMA is an independent risk factor for coronary heart disease and in critically ill patients an independent risk factor for ICU mortality (19;20).

We hypothesize that in addition to low arginine levels, premature infants with NEC have an increased plasma concentration of ADMA. Increased ADMA levels

are associated with reduced NO synthesis and may consequently lead to a decreased mucosal blood flow and barrier function of the gut.
Therefore, the primary aim of this study was to measure the arginine, ADMA, and SDMA concentrations in premature infants with NEC and in control infants without NEC. The secondary aim was to study the relation between arginine, ADMA, and SDMA concentrations and mortality in premature infants with NEC.

Methods

Study design
We performed a prospective case-control study with a 1:1 ratio of case to control subjects. The hospital ethics committee approved the study and all infants were included after written informed consent of their parents.

Patients
All infants with a gestational age <34 weeks (assessed by last menstrual period of the mother and/or ultrasound before 20 weeks of gestation) and a birth weight ≤ 2000 gram admitted to the level III neonatal intensive care unit of the VU University Medical Center or the Academic Medical Center, Amsterdam, who developed NEC, were eligible for participation in the study. Only patients with NEC ≥ grade II diagnosed between 8 am and 8 pm were included in the study. Exclusion criteria were major congenital or chromosomal anomalies.
NEC was diagnosed and classified according to the criteria of Bell (21). NEC grade I was characterized by non-specific systemic signs and abdominal signs including increased gastric residuals or abdominal distension. NEC grade II encompasses the signs of stage I, absent bowel sounds or abdominal tenderness. The abdominal radiograph shows intestinal dilation, ileus and pneumatosis intestinalis. NEC grade III is characterized by bowel perforation visualized as pneumoperitoneum on the abdominal radiograph. An independent paediatric radiologist made the diagnosis of pneumatosis intestinalis.
According to the protocol guidelines for parenteral and enteral nutrition, all infants received either breast milk with breast milk fortifier (intake about 57 mg arginine /100 ml (22), and/or a formula feeding (Nenatal Start®, Nutricia Zoetermeer, The Netherlands containing 71 mg arginine /100 ml) and/or total parenteral nutrition (a hospital pharmacy based all in one mixture, containing 102 mg arginine /100 ml). After the diagnosis NEC, a peripheral venous blood sample (0.3 mL) was collected and the, infants were treated according to the standard protocol for NEC; nothing by mouth, broad-spectrum antibiotics and parenteral feeding. If this treatment failed or in case of intestinal perforation, surgery was performed.

Control infants

For each premature infant with NEC, one premature infant without NEC was selected. The selection was based on the characteristics of the NEC patient including gestational age, birth weight, head circumference, Apgar score at 5 minutes and postnatal age at the time of the diagnosis NEC. After inclusion, a peripheral venous blood sample (0.3 mL) was collected.

Plasma analysis of arginine, ADMA and SDMA

The blood samples taken from the premature infants were immediately placed on ice and centrifuged at 3000 rpm for 10 min at 4ºC. Plasma was immediately put in liquid nitrogen, and stored at –80ºC before analysis.

The concentration of ADMA, arginine and SDMA were determined simultaneously by high-performance liquid chromatography (HPLC) as described previously (23). In brief, solid-phase extraction on polymeric cation-exchange columns was performed using monomethylarginine as the internal standard. After derivatization with ortho-phthaldialdehyde reagent containing 3-mercaptopropionic acid, analytes were separated by isocratic reversed-phase HPLC with fluorescence detection. Intra- and inter-assay coefficients of variation were better than 1.2 and 3.0%, respectively. The arginine/ADMA ratio was calculated.

Statistical analysis

Due to non-normal distribution of the amino acids, non-parametric tests were used, and data are presented as median and range. Mann-Whitney U test was used to investigate differences in plasma amino acid concentrations between the premature infants with NEC and the control group, and between surviving and non-surviving NEC patients. A p-value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Between September 2001 and October 2003, a total of 33 premature infants with NEC were eligible to enter the study. Of these 33 infants, 23 were excluded for the study (13 infants with NEC grade I and 10 infants were diagnosed and treated at night or during the weekend). The ten premature infants with NEC ≥ stage II were included in the study. Six premature infants with NEC were treated by surgery, while four infants were treated with antibiotics and parenteral nutrition only. The control group consisted of ten case controlled premature infants without NEC. Patient and nutrition characteristics are presented in Table 1. The patient characteristics were not different in the NEC and control group. Breast milk feeding, parenteral nutrition energy intake and the intake of arginine, carbohydrate, protein and fat were not different in the two groups.

Table 1. Nutritional and patient characteristics of premature infants with NEC and without NEC (controls).

	NEC (N = 10)		Control (N = 10)	
Male: Female	5 : 5		6 : 4	
Gestational age (weeks)	27.6	25.6-31.9	28.7	26.3-33.3
Birth weight (gram)	968	700-1822	1102	690-1710
Head circumference (cm)	28	22-35	28	23-30
Apgar score 5 minutes	9	3-10	8	4-9
Postnatal age (days)	16	7-38	14	7-30
Breast milk feeding	N = 4/10		N = 7/10	
Parenteral nutrition	N = 3/10		N = 0/10	
Enteral feeding	N = 9/10		N = 10/10	
Nutrition intake				
Energy (kcal/kg)	70	10-122	82	5-129
Carbohydrate (g/kg)	9	2-14	12	0.3-13
Protein (g/kg)	1.7	0.7-2.1	0.5	0-3.9
Fat (g/kg)	3.8	0.4-10.2	3.2	0.2-7.1
Arginine (mg/kg)	82	27-175	119	14-145

Data are presented as median and range.

Plasma concentrations of arginine, ADMA, SDMA and the arginine/ADMA ratio are shown in Table 2. The arginine concentration in premature infants with NEC was lower (p=0.001) compared to the infants without NEC. The ADMA concentration in infants with NEC was also lower (p=0.009) compared to the control group. Plasma SDMA was not different in the two groups. The arginine/ADMA ratio was lower (p=0.023) in the NEC group.

Table 2. Plasma concentration of arginine, ADMA, SDMA and the arginine/ADMA ratio in premature infants with and without NEC (controls).

	NEC (N = 10)		Control (N = 10)		p-value
Arginine *(µmol/L)*	21.4	11.5-59.3	55.9	27.1-122.3	p = 0.001
ADMA *(µmol/L)*	0.59	0.33-1.02	0.85	0.58-1.18	p = 0.009
SDMA *(µmol/L)*	1.07	0.78-2.88	1.39	0.74-2.05	NS
Arginine/ADMA	36.6	15.6-94.4	72.3	35.9-103.4	p = 0.023

Data are presented as median and range.

Of the 10 NEC patients, 4 patients died because of circulatory and respiratory insufficiency. In the non-surviving NEC infants, arginine and arginine/ADMA concentrations were lower (p=0.01, p=0.038 respectively) than in the surviving infants (Table 3).

Table 3. Plasma concentrations of arginine, ADMA, SDMA and the arginine/ADMA ratio in premature infants with NEC (non-survivors and survivors).

	Non-survivors (N = 4)		Survivors (N = 6)		p-value
Arginine (µmol/L)	14.7	11.5-16.4	33.8	17.7-59.3	p = 0.01
ADMA (µmol/L)	0.46	0.33-1.02	0.63	0.52-0.84	NS
SDMA (µmol/L)	0.96	0.78-2.88	1.14	0.91-1.28	NS
Arginine/ADMA	32.0	15.6-35.7	47.5	34.2-94.4	p = 0.038

Data are presented as median and range.

The plasma concentrations of the premature infants without NEC and the surviving NEC patients are shown in Table 4. The plasma concentration of arginine, ADMA and SDMA were lower in the surviving NEC patients (P=0.022, P=0.031, P=0.022 respectively). The arginine/ADMA ratio was not different between the two groups.

Table 4. Plasma concentrations of arginine, ADMA, SDMA and the arginine/ADMA ratio in surviving premature infants with NEC and without NEC (controls).

	Survivors (N = 6)		Control (N = 10)		p-value
Arginine (µmol/L)	33.8	17.7-59.3	55.9	27.1-122.3	p = 0.022
ADMA (µmol/L)	0.63	0.52-0.84	0.85	0.58-1.18	p = 0.031
SDMA (µmol/L)	1.14	0.91-1.28	1.39	0.74-2.05	p = 0.022
Arginine/ADMA	47.5	34.2-94.4	72.3	35.9-103.4	NS

Data are presented as median and range

Discussion

Our study shows that premature infants with NEC have significantly lower plasma concentrations of arginine than premature infants without NEC. The lower concentration of arginine in NEC patients is in line with the results of other studies (4;5). In addition, ADMA and the arginine/ADMA ratio are also significantly lower in premature infants with NEC. Furthermore, we found in non-surviving NEC patients a significantly lower arginine concentration and arginine/ADMA ratio than in surviving NEC patients. However, compared to the control patients, the surviving NEC patients had still significantly lower arginine and ADMA concentrations, but the arginine/ADMA ratio was not significantly different. These results indicate that NEC patients have less substrate available for NO synthesis, which is important to sustain blood flow in the gut of these premature infants. Moreover, these results suggest that a decreased availability of substrate for NO synthesis is associated with an increased risk of mortality.

In adult mammals, approximately 60% of net arginine synthesis occurs in the kidney, whereas in neonates, arginine is synthesized in the enterocytes of the

small intestine (6). Wu et al. (24), studied the arginine synthesis in the enterocytes of foetal piglets and concluded that there was little synthesis of arginine in the enterocytes of preterm piglets. This near absence of intestinal arginine synthesis in preterm piglets is in sharp contrast to the high rate of intestinal arginine synthesis in term piglets (25). In the enterocytes of preterm piglets, there is low activity of the Δ^1 –pyrroline-5-carboxylate (P5C) synthase enzyme, which may limit the conversion of glutamine into citrulline (24). Furthermore, there is negligible expression of argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). Both enzymes are essential for the conversion of citrulline into arginine. Notwithstanding that these studies are performed in animal models, decreased expression of ASS and ASL and the incapability of the foetal kidney to synthesize arginine may account for the decreased level of arginine in premature infants.

Arginine is a physiological precursor for the synthesis of NO which plays an important role in regulating vascular tone, the development of gastro-intestinal circulation, and in preserving the integrity of the gastro-intestinal mucosal barrier (6;7;26;27). Conversely, the NOS inhibitor L-NNA preferentially reduces the gastrointestinal blood flow as shown in foetal sheep (28). In a rat model, the NOS inhibitor L-NMMA enhanced intestinal damage after administration of endotoxin. However, this damage could be reversed by administration of arginine (29). The mucosal permeability of the feline ileum increased after infusion with the NO synthesis inhibitor L-NAME, while infusion with the NO donor sodium nitroprusside or arginine reversed this effect (8). These animal studies indicate that a low plasma concentration of arginine, and inhibition of the arginine-NO pathway, could contribute to decreased synthesis of NO, thereby resulting in decreased gastro-intestinal blood flow and impairment of the intestinal mucosal barrier.

Interestingly, in contrast to our hypothesis, a significantly lower ADMA concentration was found in premature infants with NEC. Because ADMA is an endogenous inhibitor of all isoforms of NOS, a decrease of ADMA in premature infants with NEC could be associated with attempted regulatory changes aimed at a preservation of NO production in the presence of low arginine concentrations.

Although we cannot designate a single mediator for the observed effect, theoretically an increased metabolic turnover of ADMA may be responsible for the lower levels in NEC patients. ADMA is eliminated from the body by renal excretion as well as metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) which is widely distributed but in particular in the pancreas, liver and kidney (30;31). Induction of this enzyme or an increased renal excretion in NEC patients could be responsible for the decreased ADMA concentration (31;32). Nijveldt et al. (10;33) showed a reduced systemic ADMA concentration in rats during endotoxemia, indicating increased DDAH activity. In addition, acute Escherichia Coli endotoxaemia in humans, decreased the plasma

arginine/ADMA ratio (34). Since arginine and other cationic amino acids such as ornithine and lysine but also ADMA and SDMA are transported into endothelial cells by CAT, changes in the expression of CAT mRNA by endotoxemia may influence ADMA transport (13). Hattori et al.(35), showed that expression of CAT 1 and 2 mRNA in the lung, heart, and kidney was increased by injection of lipopolysaccharide / Interferon gamma (LPS-IFN) in rats whereas CAT 2A mRNA was abundantly expressed in the liver independent of LPS-IFN treatment. The abundant expression of CAT 2 mRNA in the liver indicates a potentially high uptake of dimethylarginines in this organ. The important function of endotoxemia inducing DDAH, as well as the expression of the CAT, provides a possible explanation for lower ADMA concentration in NEC patients.

Although the pathophysiology of NEC is multifactorial, there is some evidence suggesting that the development of NEC is associated with intestinal gram-negative bacterial growth (1;2;36). Gram-negative bacteria produce the endotoxin lipopolysaccharide (LPS), inducing endotoxemia and the endogenous production of platelet activating factor (PAF) and tumour necrosis factor (TNF) (37-40). The combination of LPS, PAF, and TNF increases intestinal epithelial permeability and often causes necrosis which could contribute to the development of NEC (29;41). Therefore, endotoxemia due to bacterial growth and release of LPS in premature infants can not only contribute to the development of NEC but can also induce DDAH and the expression of CAT, thereby increasing elimination of ADMA.

The lower concentration of ADMA could be a compensatory mechanism to counteract the disadvantageous effect of low arginine concentrations in NEC patients. However, since the decrease of arginine is larger than the decrease of ADMA, the arginine/ADMA ratio is lower in NEC patients, suggesting a reduced capacity for NO synthesis.

In this study we did not measure the oxidation products nitrate and nitrite (NOx) as an indirect determination of endogenous formation of NO. Because the NOx concentration is influenced by endogenous NO synthesis, dietary intake but also by the excretion in urine, faeces and expired air, NOx can not be used as a truly meaningful indicator of NO production in premature infants (42).

In conclusion, the present study confirms the existence of low arginine concentrations in premature infants with NEC. In contrast to our hypothesis, the ADMA concentration is lower in these infants. In spite of the low ADMA concentration, the arginine/ADMA ratio is lower in NEC patient than in controls. Furthermore, a low arginine plasma concentration and a low arginine/ADMA ratio are associated with an increased risk of mortality in NEC patients. These findings suggest that despite the increased need for arginine, premature infants with NEC have lower availability of this substrate to synthesize NO, which may be involved in the pathophysiology of NEC. The results of our study suggest the association between arginine, ADMA and NEC. However it remains to be determined whether low levels of arginine contribute to the pathophysiology of

NEC, or if low arginine levels are a consequence of NEC. As suggested in the few other studies to date, we suggest that the supplementation of arginine may prevent the development of NEC in premature infants. Due to the low incidence of NEC (on average 6 per 300 patients/year), and a high local nosocomial infection rate in our center (43) a multicenter randomised controlled study of arginine supplementation in premature infants is needed to determine whether arginine supplementation reduces the development of NEC.

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