THE ROLE OF OXIDATIVE STRESS IN THE NECROTIZING ENTEROCOLITIS OF PRETERM

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Abstract. Introduction: Oxidative stress (OS) is strongly envolved in the pathogenesis of many preterm newborn diseases; this is due to the low efficiency of natural antioxidant systems unable to counteract the harmful effects of free radicals (FRs). Necrotizing Enterocolitis (NEC) is a multifactorial disease and it is part of the so called "free radicals related diseases". Hypoxic-ischaemic events and inflammation, involved in NEC pathogenesis, are responsible of the overproduction of free radicals (FRs), generating OS.
Aim: To test the hypotesis that OS marker levels in cord blood may predict the onset of NEC in high risk infants.
Materials and methods: 91 preterm newborns of gestational age between 24 and 33 weeks and birth weight between 460 and 2540 grams were consecutively recruited in two italian neonatal intensive care units. Markers of potential oxidative stress risk, Non Protein Bound Iron (NPBI), and free radicals damage, Advanced Oxidation Protein Products (AOPP) and Total Hydroperoxide (TH), were measured in the cord blood. Associations between NEC and OS markers have been checked through inferential analysis (univariate logistic regression).
Results: Out of 91 preterm babies, 8 developed NEC. Babies with NEC had a birth weight (BW) and a gestational age (GA) significantly lower than healthy babies (BW=1100,52 ± 444,30 vs 1320,53 ± 462,09; GA=29,02 ± 2,09 vs 30,14 ± 2,33, respectively. $p<0.005$).
Cord blood levels of TH and NPBI were higher in babies with NEC than in babies without, but not significantly. AOPP cord blood levels were significantly higher in babies with NEC than the babies without (AOPP=29,15 ± 20,02 vs 16,72 ± 7,34; $p<0.05$). AOPP demonstrated a significant value for the identification of the risk of NEC (OR=1.13, CI 95%= 1.001-1.282).
Conclusions: OS is strongly associated with NEC. The determination of biochemical OS markers in cord blood can be useful in identifying babies at high risk to develop NEC and in devising new strategies to bring consistent benefits to premature babies.
Keywords: oxidative stress, necrotizing enterocolitis, free radicals, preterm babies.

INTRODUCTION

Oxidative stress (OS) is one of the main mechanism involved in the pathogenesis of many diseases of newborn. A physiological oxidative stress occurs at birth in all newborns as consequence of the hyperoxic challenge due to the transition from the hypoxic intrauterine environment to extrauterine life.
In preterm babies low efficient antioxidant systems are not able to counteract the harmful effects of reactive oxygen species, leading to free radicals disease of newborns involving serious cellular, tissue and organ damages (kidney, retina, lung and bowel injury) (1).
Necrotizing enterocolitis (NEC) is a gastrointestinal surgical emergency in premature low birth-weight neonates (2). The overall incidence is 1–5 per 1000 live births, dramatically increasing to 100 per 1000 in very low birth weight and to 200 per 1000 in extremely low birth weight infants. Death occurs in 25–30% with 25–50% of survivors requiring surgical intervention to remove affected intestinal sections (3).
The etiology of NEC is multifactorial: ischemia, bacteria, cytokines, enteral feeding and oxidative stress may contribute to the disruption of the protective gut barrier, even if pathogenesis is currently unclear.
In particular, a potential link between reactive oxygen species (ROS) produced by ischemia-reperfusion injury to the premature gut and the development of NEC has been described (4-6).
In our study we measured levels of OS biomarkers in cord blood of preterm babies in order to correlate perinatal OS with NEC. This study tests the hypothesis that OS biomarkers levels in cord blood may predict the onset of NEC in high risk infants.

MATERIALS AND METHODS

Patients

Ninety-one preterm newborns of gestational age between 24 and 33 weeks and birth weight between 460 and 2540 grams (g) were consecutively recruited from 2 italian neonatal intensive care units: Siena and Milan (for more details see Table 1).
Inclusion criteria were at least one of the following: gestational age less than 34 weeks and birth weight

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Table 1. Clinical characteristics of enrolled patients.

	NEC	NO NEC
Patients n°	**8**	**83**
Gestational Age week (mean±SD)	**29,02 ± 2,09**	**30,14 ± 2,33**
Birth Weight grams (mean±SD)	**1100,52 ± 444,30**	**1300,53 ± 462,09**
Apgar Score at 1 min (mean±SD)	**6,3 ± 0,8**	**7,4 ± 0,2**
Apgar Score at 5 min (mean±SD)	**7,8 ± 0,3**	**8,2± 0,7**
pH at 1h of life (mean±SD)	**7,21 ± 0,3**	**7,28 ± 0,2**
Mode of deliver		
• **VD**	**1**	**44**
• **ECS**	**3**	**32**
• **UCS**	**4**	**7**
Sex		
• **Male**	**3**	**48**
• **Female**	**5**	**35**
ABE at 1h of life (mean±SD)	**-8 ± 3**	**-3 ± 2**

less than 2600 g.
Newborns with major congenital malformations, inborn errors of metabolism and blood group incompatibility were excluded from the study.
The diagnosis of NEC was based on clinical and radiological findings.
Clinical symptoms and findings such as increased episodes of apnea and desaturation, bradycardia, lethargy, temperature instability, feeding intolerance, vomiting, increased gastric residual volume (> 20%), bilious or bloody gastric aspirate, decreased bowel sounds, bloody stools, abdominal distension and tenderness, and abdominal wall skin color changes were evaluated. Infant with two or more of these symptoms/findings were subjected to laboratory and radiographic evaluations.
Radiographic abnormalities enclosed air-fluid levels, dilated and gas-filled loops of bowel and thickened bowel walls, pneumatosis intestinalis, portal venous gas, ascites and pneumoperitoneum. A complete blood count, blood smear, levels of IL-6 and C-reactive protein, serum electrolytes, serum urea nitrogen, creatinine, liver function tests, urinalysis and blood cultures were tested.
The occurrence of NEC was diagnosed according to modified Bell criteria (7,8).

Methods
Heparinized blood samples were drawn from the umbilical vein after cord clamping immediately after delivery. Complete blood cell count and gas analysis were performed. Blood samples were stored up at -80 °C and afterwards analyzed.
Marker of OS potential risk, Non Protein Bound Iron (NPBI [µmol/L]) and markers of OS-linked damage: Total Hydroperoxides (TH [UCARR/L]) and Advanced Oxidation Protein Products (AOPP [µmol/L]) were assessed.
Blood gas analysis was performed with a Radiometer ABL 505 analyser (Radiometer, Copenhagen, Denmark) immediately after blood sampling. The blood was centrifuged and plasma and buffy coats were removed. NPBI plasma levels were detected using the method described by Paffetti and colleagues (9). TH production was measured with the d-ROMs Kit (Diacron Srl, Grosseto, Italy) (10) and AOPP were measured as described by Witko-Sarsat and 4 colleagues using spectrophotometry on a microplate reader (11).

Statistical analysis
Associations between NEC presence/absence and the OS markers were analyzed first through descriptive and then inferential analysis. Summary statistics of data were expressed as mean ± standard deviation (SD), minimum and maximum for descriptive analysis of continuous variables, whereas for categorical variables were reported absolute frequencies.
Logistic univariate regression was chosen as inferential analysis and it was inquired the relation between the presence/absence of NEC as a dependent variable, and each OS marker as an independent variable. Through the logistic model it is possible to calculate estimated coefficients (B), standard error (S.E.), Wald statistic, Significance (P) and exponential or odds ratio Exp(B) with the relative confidence interval (95% CI). A p-value less than 0.05 was considered significant. The above tests were performed using the SPSS V.16 for Windows statistical package (SPSS Inc, Chicago, IL, USA).

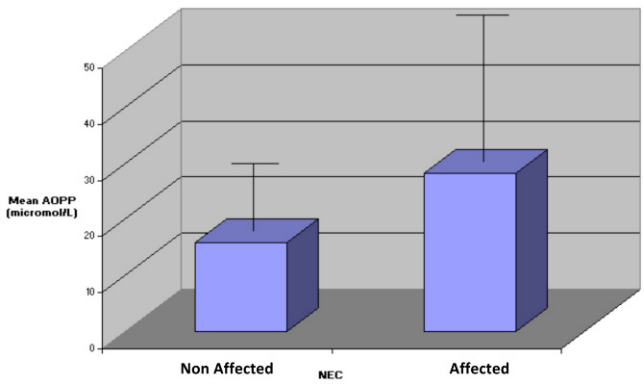
RESULTS
Our study population, consisting in 91 infants, had a gestational age (GA) of 24-33 weeks (30,12 ± 1.16)

Table 2: Oxidative stress markers vs presence/absence of NEC

Ox stress biomarkers	NEC	NO NEC	p
AOPP (µmol/L)	29,15 ± 20,02	16,72 ± 7,34	<0,05
TH (UCARR/L)	167,32 ± 88,44	123,98 ± 87,63	NS
NPBI (µmol/L)	2,38 ± 5,75	1,51 ± 3,54	NS

with the following percentage distribution: 54,33% between 27-30 weeks, 34,52% between 31-33 weeks, 11,06 % between 24-26 weeks. The birth weight (BW) was between 460-2540 g (1124,73 ± 251,01) with the following percentage distribution: 1,16% less than or equal to 500 gr, 7,10% between 501-750 g, 24,56% between 751-1000 g, 67,18% more or equal 1001 g.
Out of 91 preterm babies, 8 developed NEC (all stages). Babies with NEC had BW and GA significantly lower than healthy babies (BW=1100,52 ± 444,30 vs 1320,53 ± 462,09; GA=29,02 ± 2,09 vs 30,14 ± 2,33, respectively. p<0.005).
Cord blood levels of TH and NPBI were higher in babies with NEC than in babies without, but not significantly (Table 2).
AOPP cord blood levels were significantly higher in babies with NEC than the babies without (AOPP=29,15 ± 20,02 vs 16,72 ± 7,34; p<0.05) (Fig. 1).

Fig.1. *Mean AOPP vs presence/absence of NEC*



The inferential analysis showed that the development of NEC is significantly associated to the presence of high AOPP values in cord blood (OR=1.13, CI 95%= 1.001-1.282) (Table 3).

Discussion

The relationships between FRs production and tissue damage are very complex in perinatal period. FRs are produced by various mechanisms such as Fenton reaction, purine and mithocondrial metabolism, phagocytic activation during inflammatory or hypoxic-ischemic processes. Toxic molecules produced by these different mechanisms are involved in the pathogenesis of some newborn diseases. Previous studies demonstrated that excessive FRs production is largely responsible of cellular, tissue and organ damage (12,13).
When OS occurs in intrauterine life, it may represent a risk factor for the development of neonatal pathologies. (1)
NEC is due to a wide range of mechanisms, such as inflammation, oxidative stress and activation of apoptotic signaling in intestinal epithelial cells during perinatal injury. Reactive oxygen species (ROS), generated as a result of ischemia-reperfusion injury to the gut, have been linked to the development of NEC in premature infants (14,15). Cytokines are also thought to play a role in ROS generation, contributing to severe gut inflammation and injury during NEC caused by the exaggerated inflammatory responses by the premature immune system (16,17).
Preterm infants are more susceptible to OS damage than term infants due to organs' structural and functional immaturity, overloading of aerobic tissues metabolism with rapidly growing energy demand, conditions leading to excessive FRs production (hypoxia-ischemia, hyperoxia, infections), presence of high NPBI levels, lack of antioxidant systems that come to maturity during the first year of life (18-21).
We measured OS biomarkers in cord blood as indicators of fetal exposure to intrauterine production of FRs, leading to the onset of NEC, as FRs mediated disease in premature infants.
We found that babies with NEC had higher values of

Table 3. Logistic regression analysis results. AOPP vs presence/absence of NEC

	B	S.E.	Wald	P	OR	95,0% C.I
AOPP	0,125	0,063	3,920	0,048	1,133	1,001-1,282

cord blood TH, AOPP and NPBI than normal babies. NPBI, being redox cycling active, is an OS biomarker. It has pro-oxidant properties because it can enter in Fenton reaction producing hydroxyl radical, the most oxidant molecule in biological systems.

TH, are indexes of overall FRs attack, because they are indicative of intermediate oxidative products of lipids, peptides and aminoacids. Lipid and protein hydroperoxides, in the presence of traces of free iron, produce several secondary reactive radical species which can be measured collectively as organic hydroperoxides (10). AOPP are terminal products of protein exposure to FRs without oxidant properties and they are reliable markers of the degree of protein damage in oxidative stress (10).

It is interesting to note that among OS biomarkers, AOPP had significant prognostic value for the risk of developing NEC.

When an intrauterine OS occurs, proteins are the main and early target of oxidation in plasma (22). In previous our study we demonstrated an association between NPBI and carbonylated proteins in babies with highest NPBI levels. Since NPBI may produce hydroxyl radicals through the Fenton reaction, the major target of OS induced by NPBI is its carrier: albumin (23).

Oxidation of protein can therefore be expected to increase the likelihood of tissue damage due to OS in the newborn.

Our results underline a strong association between NEC and AOPP, showing a clear correlation between intrauterine OS events and the risk of developing NEC. The use of cord blood samples shows that NEC is a perinatal disease linked to FR-mediated injury originating in intrauterine life.

This finding of increased plasma levels of OS biomarkers in newborn who developed NEC suggests that increased OS markers are a direct index of increased production of FRs in the premature gut as a response to oxidative damage. This hypothesis is reinforced by the observation that similar increased FRs production occurs after perinatal injury (24).

We conclude that the determination of biochemical OS biomarkers in cord blood allows the early identification of infant at risk for NEC and can be useful in devising new strategies to bring consistent benefits to premature babies.

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