DOI: 10.4081/jsas.2013.49

FREE RADICAL-RELATED DISEASES: THE PREDICTIVE VALUE OF BIOMARKERS IN THE UMBILICAL CORD BLOOD

S. Perrone, M.G. Alagna, M.S. Cori, A. Santacroce, S. Negro, M.L. Tataranno, M. Longini, C. Felici, F. Proietti, A. Picardi, G. Buonocore

Department of Developmental and Molecular Medicine, General Hospital "Santa Maria alle Scotte", University of Siena, Siena, Italy

Abstract. Despite recent advances in preterm newborns healthcare, the incidence of neonatal pathologies and disabilities still remain unacceptable high. The deficiency of antioxidant
systems and the high free radicals (FRs) production may cause several neonatal diseases, such as Retinopathy of Prematurity (ROP), Bronchopulmonary Dysplasia (BPD), Necrotizing Enterocolitis (NEC), Patent Ductus Arteriosus (PDA), Periventricular Leukomalacia (PVL) and Intraventricular Hemorrhage (IVH), representing facets of the 'Free Radical-Related Diseases' (FRD). The aim of this study is to verify the association between FRD and blood levels of reliable oxidative stress (OS) biomarkers in preterm newborns. We enrolled 178 preterm newborns born consecutively at the General Hospital "Santa Maria alle Scotte" in Siena, between 23 and 34 weeks (30,36±2.97) of gestational age, with birth-weight from 430 to 2890 grams (1453±593). After birth, we evaluated in the cord blood the markers of potential risk of OS (Non Protein-Bound Iron, NPBI) and the markers of FR damage (Total Hydroperoxides, TH; Advanced Oxidation Protein Products, AOPP). For each newborn, we assessed the presence or absence of the following diseases, considering as FRD the presence of one at least: ROP, BPD, NEC, PDA, PVL, IVH. The univariate logistic regression showed a significant association between FRD and OS related markers. Risk assessment of FRD was higher in newborns with higher values of each OS marker: respectively TH (OR=1.013, p=0,000), AOPP (OR=1.017, p=0,036), NPBI (OR=1.077, p=0.039). Perinatal OS exposure is linked to the main diseases of prematurity. The evaluation of OS biomarkers in preterm newborns through the analysis of umbilical cord blood, can be useful and predictive for early identification of infants at risk for FRD in order to devise appropriate and timely prevention and treating strategies.

Key words: preterm newborns, oxidative stress, free radical-related diseases, biomarker.

Abbreviations: Free Radicals (FRs), Free Radical-Related Diseases (FRD), Retinopathy of Prematurity (ROP), Bronchopulmonary Dysplasia (BPD), Necrotizing Enterocolitis (NEC), Patent Ductus Arteriosus (PDA), Periventricular Leukomalacia (PVL), Intraventricular Hemorrhage (IVH), Oxidative Stress (OS), NonProtein-Bound Iron (NPBI), Total Hydroperoxides (TH), Advanced Oxidation Protein Products (AOPP).

BACKGROUND

Preterm birth can result in significant developmental disability and several studies have identified Retinopathy of Prematurity (ROP), Bronchopulmonary Dysplasia (BPD), Necrotizing Enterocolitis (NEC), Patent Ductus Arteriosus (PDA), Periventricular Leukomalacia (PVL) and Intraventricular Hemorrhage (IVH) as major causes of adverse outcome for preterm neonates [1-3].

Preterm birth represents a unique event for the developing fetus and many important environmental factors, including inflammation, hypotension and hypoxemia contributing to morbidity, have been identified. Following hypoxia, hyperoxia, ischemia and inflammation, a cascade of several biochemical events occurs and the endpoint is free radicals (FRs) overproduction [4-10]. Iron ions, serving as transition metal molecules catalyzing hydroxyl radical production via the Fenton reaction and the Haber-Weiss cycle, accumulate in cells, after the occurrence of transient hypoxia-ischemia. Iron and FRs may result in DNA strand breaks[11], protein and lipid peroxidation[12], cellular inflammation[13] and death[14,15].

In preterm newborns, the overproduction of FRs and the insufficiency of antioxidant mechanisms result in oxidative stress (OS). OS induces cellular, tissue and organ impairment due to the disruption of physiological balance between pro-oxidant and anti-oxidant agents [16].

The sequelae of oxidative injury include conditions such as of ROP, PVL, BPD, NEC, PDA and IVH [17-24], representing different facets of the "Free Radical Related Disease" (FRD). Unfortunately, despite the improvement in perinatal care and survival of premature infants,

Correspondence to:
Dott.ssa Serafina Perrone
Department of Molecular and Developmental Medicine
University of Siena, Policlinico Santa Maria alle Scotte.
Viale Bracci 36, 53100 Siena, Italy
E-mail: saraspv@yahoo.it

the incidence and severity of these diseases is still high.

FRD prevention and early diagnosis, through the detection of specific biomarkers, are useful to improve short and long term outcomes in premature infants.

This study tests the hypothesis that levels of OS biomarkers early assessed in cord blood of preterm newborns may predict the later onset of FRD.

METHODS

One hundred and eighty-nine preterm newborns born consecutively at the General Hospital "Santa Maria alle Scotte" in Siena from 1/1/2006 to 1/1/2009 were enrolled. Newborns with major congenital malformations, inborn errors of metabolism, incompatibility group were excluded from the study. Eleven babies died soon after birth. Thus the final neonatal cohort consisted of 178 babies with a gestational age (GA) between 23 and 34 weeks (mean: 30,36±2,97) and birth weight (BW) between 430 and 2890 grams (mean: 1453±593). Heparinized blood samples were drawn from the umbilical vein after cord clamping immediately after delivery. Complete blood cell count and gas analysis were performed. Variables selected for statistical evaluation were: GA; BW; presence/absence and type of pathology (IVH, BPD, PDA, PVL, NEC and ROP); markers of OS potential risk non-protein bound iron, NPBI) and markers of OS-linked damage (total hydroperoxides, TH; advanced oxidation protein products, AOPP) were assessed. NPBI, being redox cycling active, has pro-oxidant properties because it can enter in Fenton reaction producing hydroxyl radical, the most oxidant molecule in biological systems. TH are indices of overall FRs attack, because they are indicative of intermediate oxidative products of lipids, peptides and amino acids. AOPP are terminal products of protein exposure to FRs without oxidant properties [25].

To avoid storage effects, all analyses were carried out in plasma within 2 h of blood sampling. NPBI plasma levels were detected by HPLC using the method described by Kime and colleagues, but with some modifications[25]. NPBI and AOPP assays were performed in duplicate to avoid methodological variations. AOPP were measured as described by Witko-Sarsat and colleagues using spectrophotometry on a microplate reader. TH production was measured with the d-ROMs Kit (Diacron Srl, Grosseto, Italy) [25].

Brain ultrasonography was performed within 48 h after birth with real-time ultrasound machine with transducer frequency emission of 7.5 mHz (Interspec Apogee, Milan, Italy) in all preterm newborns. The scans were repeated serially according to clinical conditions and at least every 5 or 6 days until a mean postmenstrual age of 40 weeks to check for IVH according to Perlman *et al.*[26]. The occurrence of IVH was diagnosed according to Papile *et al.* [27]. Ophthalmoscopic evaluations were scheduled in all infants with less than 32 weeks and in all who needed O2 therapy; checks were then repeated weekly until retinal maturation. Bronchopulmonary dysplasia was defined as the need for supplemental oxygen at 36 weeks' postmenstrual age, in agreement with the new classification system [28]. The NEC diagnosis was based on the discovery of some radiographic findings such as intestinal dilation, ileus and intestinal pneumatosis, in combination with clinical and laboratory signs and symptoms [29]. Clinical management was the same for all infants. Clinicians were blind to the laboratory test results.

Statistical analysis

Associations between FRD presence/absence and the OS markers were analyzed first through descriptive and then inferential analysis. Summary statistics of data were expressed as mean (with SD), minimum and maximum for descriptive analysis of continuous variables, whereas categorical variables were reported absolute frequencies (Table 1). Logistic univariate regression was chosen as inferential analysis, instead of multivariate logistic regression for the lack of data, and the relation between the presence/absence at least one of FRD as a dependent variable and each OS marker as an independent variable was checked. Through the logistic model it is possible to calculate significance (*p value*) and exponential or odds ratio (Exp B). A *p-value* less than 0.05 was considered significant. This was useful to show if the increase of OS

Table 1. Characteristics of study-group.

	Discharge out come	Mean±SD	Range (min-max)
GA (weeks)	Normal and affected infants	30,36±2.97	23-34
Birth weight (g)	Normal and affected infants	1453.11±593.54	430-2890
AOPP (µmol/L)	Affected infants	17,192±11,1054	0-43
	Normal infants	11,181±5,8186	0-26
TH (UCARR/L)	Affected infants	105,58±89,525	15-333
	Normal infants	81,44±34,54	8-161
NPBI (µmol/L)	Affected infants	4,6732±5,79042	0-28,98
	Normal infants	2,1503±3,58823	0-26,21

marker values in cord blood was a risk factor for the development of such pathologies. The above tests were performed using the SPSS V.20 for Windows statistical package (SPSS Inc, Chicago, IL, USA).

RESULTS

A summary of study-group characteristics with concentration mean of OS biomarkers in sick and healthy newborns has been reported in Table 1.

Table 2 shows frequencies and relative percentages of each disease considered within FRD, defined as the presence of at least one of such pathologies.

Descriptive analysis showed that affected babies had higher means of TH, AOPP and NPBI (Table 1). Univariate logistic regression gives significant *p* values for each marker we evaluated (NPBI: p= 0,039, OR=1,077; AOPP: p=0,036, OR=1,017; TH: p=0,000, OR=1,013). Especially, NPBI seems to be the best predictor marker of OS (Table 3).

DISCUSSION AND CONCLUSIONS

Although neonatal pathologies have multiple causes, FRs damage is widely recognized as main aethiopathogenic mechanism[5,6].

Table 2. FRD frequencies and respective percentages in study population.

Disease	Number of cases (%)
NEC	7 (3,9%)
ROP	16 (9%)
PDA	45 (25,3%)
BPD	18(10,1%)
PVL	8 (4,5%)
IVH 1°	26 (14,6%)
IVH 2°	6 (3,4%)
IVH 3°	9 (5,1%)

Table 3. Inferential analysis results: *p values* and ORs for each markers evaluated.

Marker	*P value*	Odds ratio
NPBI	0,039	1,077
AOPP	0,036	1,017
TH	0,000	1,013

Oxygen and derived metabolites, collectively termed reactive oxygen (ROS) and reactive nitrogen species (RNS), are persistently produced in aerobic organisms.

When generated in excess, ROS mutilate molecules and are important mediators of cell and tissue damage [30,31].

FRs are highly unstable and normally their formation is controlled by several beneficial compounds known as antioxidants; these protective molecules are part of the antioxidant defence system. There is a critical balance between FRs generation and antioxidant defences: when FRs production is exacerbated or scavenging insufficient, dysregulation of many biological processes occurs. FRs lead to the oxidation of lipids, proteins, polysaccharides and to DNA damage (fragmentation, base modifications and strand breaks); as a consequence, radicals have a wide range of biologically toxic effects. Newborns, especially if born prematurely, are very susceptible to free radical oxidative damage, for many reason: (a) infants at birth are naturally exposed to the hyperoxic challenge due to the transition from the hypoxic intrauterine environment to extrauterine life and this gap is even more significant for newborns that require supplemental oxygen during resuscitation in the delivery room; (b) they are more susceptible to infection, especially if born prematurely; (c) the antioxidant defences are reduced ; (d) the high levels of free iron enhances the Fenton reaction causing the production of highly toxic radicals [24]. OS likely contributes to the severity of several newborn diseases, that represent facets of "Free radical diseases of neonate." The concept implies that OS affects a variety of organs, often simultaneously, and gives rise to different signs according to the organ most damaged [32-34]. This means that the above-mentioned conditions are not different disease entities but are simply different organ manifestations of the same complex processes of OS and metabolism.

Previous our studies showed that intrauterine growht restriction, low birth weight, premature rupture of membranes and preterm delivery itself may occur as consequence OS injury, resulting as an early exposure to FRs during pregnancy[35]. Moreover, a direct relationship between hypoxia levels and FRs production in fetal life has been demonstrate[4,36].

In this study we found a significant association between OS biomarkers and neonatal diseases, showing that OS is an important risk factor for neonatal FRD. In particular, our data showed that the risk of developing FRD is increased in babies with high levels of TH, AOPP and above all NPBI in cord blood. The evaluation of samples from the umbilical cord blood at birth is meaningful, showing that neonatal pathologies are linked to FRs injury already in womb.

Especially, we pointed out the stronger association between FRD and NPBI levels than other biomarkers, underlining the role of NPBI as the best predictor of OS.

Therefore, the evaluation of OS biomarkers assessment can be considered as an useful device in the early

identification of FRD risk in newborns, in order to allow appropriate treatments and timely prevention strategies.

REFERENCES

1. *Hintz SR, Kendrick DE, Vohr BR, et al. Changes in neurodevelopmental outcomes at 18 to 22 months' corrected age among infants of less than 25 weeks' gestational age born in 1993-1999. Pediatric. 2005;115:1645-51.*
2. *Stoll BJ, Hansen NI, Bell EF, et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. Pediatrics 2010;126:443-56.*
3 *Berkowitz GS, Papiernik E. Epidemiology of preterm birth. Epidemiol Rev 1993;15:414-43.*
4. *Mishra OP, Delivoria-Papadopoulos M. Cellular mechanisms of hypoxic injury in the developing brain. Brain Res Bull 1999;48:233-8.*
5. *Yoon BH, Romero R, Jun JK, et al. Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. Am J Obstet Gynecol 1997;177:825-30.*
6. *Andrews WW, Goldenberg RL, Faye-Petersen O, et al. The Alabama Preterm Birth study: polymorphonuclear and mononuclear cell placental infiltrations, other markers of inflammation, and outcomes in 23- to 32-week preterm newborn infants. Am J Obstet Gynecol 2006;195:803-8.*
7. *Blumberg RM, Cady EB, Wigglesworth JS, et al. Relation between delayed impairment of cerebral energy metabolism and infarction following transient focal hypoxia-ischaemia in the developing brain. Exp Brain Res 1997;113:130-7.*
8. *Ciccoli L, Rossi V, Leoncini S, et al. Iron release in erythrocytes and plasma non protein-bound iron in hypoxic and non hypoxic newborns. Free Radic Res 2003;37:51-8.*
9. *Frosali S, Di Simplicio P, Perrone S, et al. Glutathione recycling and antioxidant enzyme activities in erythrocytes of term and preterm newborns at birth. Biol Neonate 2004;85:188-94.*
10. *Vento M, Asensi M, Sastre J, et al. Six years of experience with the use of room air resuscitation of asphyxiated newly born term infants. Biol Neonate 2001;79:261-7.*
11. *Barreto G, Madureira D, Capani F, et al. The role of catechols and free radicals in benzene toxicity: an oxidative DNA damage pathway. Environ Mol Mutagen 2009;50:771-80.*
12. *Adibhatla RM, Hatcher JF. Phospholipase A(2), reactive oxygen species, and lipid peroxidation in CNS pathologies. BMB Rep 2008;41:560-7.*
13. *Gongora MC, Lob HE, Landmesser U, et al. Loss of extracellular superoxide dismutase leads to acute lung damage in the presence of ambient air: a potential mechanism underlying adult respiratory distress syndrome. Am J Pathol 2008;173:915-26.*
14. *Kondo M, Yoshikawa T, Naito Y. Basic and clinical study of reactive oxygen species. Nihon Naika Gakkai Zasshi 1995;84:1186-91.*
15. *Wang D, Fasciano S, Li L. The interleukin-1 receptor associated kinase 1 contributes to the regulation of NFAT. Mol Immunol 2008;45:3902-8.*
16. *Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd ed. Oxford: Oxford Science Publications; 1999.*
17. *Halliwell B, Gutteridge JMC. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 1984;219:1-14.*
18. *Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am Rev Respir Crit Care Med 2001;163:1723-9.*
19. *Perrone S, Vezzosi P, Longini M, et al. Biomarkers of oxidative stress in babies at high risk for retinopathy of prematurity. Front Biosci (Elite Ed) 2009;1:547-52.*
20. *Epelman M, Daneman A, Navarro OM, et al. Necrotizing eneterocolitis: review of state of the art imaging findings with patologic correlation. Radio Graphics 2007;27:285-305.*
21. *Buonocore G, Perrone S, Longini M, et al. Non protein bound iron as early predictive marker of neonatal brain damage. Brain 2003;126:1224-30.*
22. *Perrone S, Tataranno ML, Stazzoni G, Buonocore G. Biomarkers of oxidative stress in fetal and neonatal diseases. J Matern Fetal Neonatal Med 2012;25:2575-8.*
23. *Saugstad OD. Update on oxygen radical disease in neonatology. Curr Opin Obstet Gynecol 2001;13:147-53.*
24. *Saugstad OD. Oxidative stress in the newborn - a 30-year perspective. Biol Neonate 2005;88:228-36.*
25. *Paffetti P, Perrone S, Longini M, et al. Non-protein-bound iron detection in small samples of biological fluids and tissues. Biol Trace Elem Res 2006;112:221-32.*
26. *Perlman JM, Rollins N. Surveillance protocol for the detection of intracranial abnormalities in premature neonates. Arch Pediatr Adolesc Med 2000;154:822-6.*
27. *Papile LA, Burstein J, Burstein R, et al. Relationship of intravenous sodium bicarbonate infusions and cerebral intraventricular hemorrhage. J Pediatr 1978;93:834-6.*
28. *Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am Rev Respir Crit Care Med 2001;163:1723-9.*
29. *Epelman M, Daneman A, Navarro OM, et al. Necrotizing eneterocolitis: review of state of the art imaging findings with patologic correlation. RadioGraphics 2007;27:285-305.*
30. *Halliwell, B. Free radicals, antioxidants and human disease: curiosity, cause, or consequence? Lancet 2009;344:721-4.*
31. *Fridovich, I. Oxygen toxicity: a radical explanation, J Exp Biol 1998;201:1203-9.*
32. *Clyman RI, Saugstad OD, Mauray F. Reactive oxygen metabolites relax the lamb ductus arteriosus by stimulating prostaglandin production. Circ Res 1989;64:1-8.*
33. *Archer SL, Peterson D, Nelson DP, et al. Oxygen radicals and antioxidant enzymes alter pulmonary vascular reactivity in the rat lung. J Appl Physiol 1989;66:102-11.*
34. *Sanderud J, Bjøro K, Saugstad OD. Oxygen radicals stimulate thromboxane and prostacyclin synthesis and induce vasoconstriction in pig lungs. Scand J Clin Lab Invest. 1993;53:447-55.*
35. *Longini M, Perrone S, Kenanidis A, et al. Isoprostanes in amniotic fluid: a predictive marker for fetal growth restriction in pregnancy. Free Radic Biol Med 2005;38:1537-41.*
36. *Mishra OP, Delivoria-Papadopoulos M. Lipid peroxidation in developing fetal guinea pig brain during normoxia and hypoxia. Dev Brain Res 1989;45:129-35.*