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Biomarkers of oxidative stress in babies at high risk for retinopathy of prematurity

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1. ABSTRACT

Oxygen-induced oxidative stress (OS) has damaging effects in the perinatal period. For now there is a lake of evidence that OS occurs in babies with retinopathy of prematurity (ROP) We tests the hypothesis that a strict oxygen policy may minimize postnatal OS reducing severity of ROP. Multicenter prospective cohort study (72 newborns), using a common clinical management protocol with a strict control of oxygen administration. Assessment of biochemical markers of OS in blood samples at birth and on days 7, 14, and 21. Sixteen babies (22.2%) developed ROP stage 1-2. No severe form of ROP was observed. Birth weight and O_2 administration in delivery room were the factors significantly associated with the development of ROP stage 1-2. Prematurity and O_2 administration in delivery room are the main factors coming into play in the course of ROP. Because room air is richer in oxygen than intrauterine environment, higher OS can be minimized, as well as incidence and severity of ROP, using standardized management with a restricted oxygen breathing policy.

2. INTRODUCTION

In developed countries, retinopathy of prematurity (ROP) is an important cause of blindness in infancy. ROP increases in incidence with decreasing gestational age and birth weight: approximately 65% of infants with a birth weight below 1,250 g develop some degree of ROP (1). Much evidence has correlated this disease with oxidative stress (OS), caused by the harmful effect of free radicals (FR) on tissues (2-4).

The dangerous effects of FR are linked to their property of being very unstable molecules and their ability to react with polyunsaturated fatty acids of cell membranes, proteins, polysaccharides, nucleic acids, causing functional alterations within the cell and DNA damage, with subsequent tissue injury (5,6). OS occurs when the production of FR overcomes the antioxidant power. Many events (hypoxia, hyperoxia, inflammation) leading to overproduction of FR may easily induce OS in the earliest phases of human life (7). FR are characterized by a very

short half-life thus they are extremely difficult to detect. Normally we measure the products of FR attacks to lipid, protein, DNA: among others isoprostanes, hydroperoxide, protein carbonyls, nitrotyrosine, dityrosine, 8-hydroxy-2'-deoxyguanosine or non protein bound iron (NPBI) as reliable markers of impending OS.

Previously we demonstrated the damaging effects of FR in the perinatal period, by evaluating several biochemical markers of OS such as total hydroperoxides (TH), advanced oxidative protein products (AOPP) and carbonilated proteins (8-12). We found an increased concentration of TH and AOPP in hypoxic preterm newborns, and a direct relation between the degree of hypoxia and the severity of oxidative damage in plasma at birth was observed (8). The OS still persisted on the seventh day of life (9). We also found an association between NPBI and carbonylated proteins in babies with highest NPBI levels confirming that plasma proteins are among the main targets of OS induced by NPBI (12).

Considering the growing role of OS in newborn preterm morbidity, one of the goals of the modern neonatology is to minimize FR production or promote the development of adequate antioxidant systems. Besides other causes of OS, an important FR source is hyperoxia with the use of supplemental oxygen for resuscitation and mechanical ventilation (13). The original cooperative trial of oxygen reduction published in 1956 demonstrated that 4 weeks of high oxygen levels would greatly increase the incidence of ROP (14). Since then, it has been assumed that the injury is always an oxidant injury and attention has focused on tighter control of oxygen administration, exogenous antioxidant administration or reduction of light exposure, which may increase the formation of oxygen FR (15-19). Although prematurity as well as oxygen have been already reported among the factors capable of inducing ROP there is yet a lake of evidence that OS occurs in babies with ROP (20). The relationships between plasma markers of OS and retinopathy remain undemonstrated.

The present study tests the hypothesis that a strict oxygen policy may minimize postnatal OS reducing incidence and severity of ROP.

3. METHODS

A prospective cohort study design was used, with data collected from a common register, through multicenter study. Eligible subjects were live-born infants with gestational age (GA) below 29 weeks and/or birth weight below 1250 g. Seven two VLBW babies born in five hospitals were enrolled in the study. Their mean GA was 27.02 +/- 1.48 (range: 24-29) weeks of pregnancy, and the mean birth weight was 856.86 +/- 183.55 g (range: 460-1240) For clinical characteristics see table 1.

The study was approved by the Human Ethics Committee of the all participant centers. Informed written parental consent was obtained before enrolment of each infant.

Common standards of care in the management of high risk newborns were established. Particular attention was paid to establishing the best values regarding vital parameters, such as blood gas analysis, transcutaneous PaO_2 and $PaCO_2$ monitoring, ventilatory setting and pulmonary compliance. The reason for choosing a tight protocol of oxygen administration and control of oxygen saturation was to avoid conditions recognized as main factors in inducing OS: hypoxia, hyperoxia, as well as sharp variations of these parameteres.

Continuous recording of transcutaneous PaO_2 was performed, setting up the pulse oximetry alarms over 84% and below 96%, since delivery room. At birth, restricted oxygen breathing policy was conducted (21). When oxygen was used, it was used only to restore blood oxygen levels to the recognized range (Sat O_2 = 84% -96%) and was stopped as soon as it was no longer needed to alleviate hypoxia.

Upon the neonates' admission to intensive therapy, particular attention was paid to tighter control of oxygen administration, reduction of light exposure, meticulous control of transcutaneous oxygen, and monitoring of blood preassure and blood gases. Early nasal continuous airway pressure (nCPAP), transcutaneous monitoring and minimal handling was applied to stabilize the functional residual lung volume and to reduce stresses and disturbances in order to enable infants to breath on their own.

In addition, a common computerized data sheet was kept for the description of clinical and biochemical data. Clinical information recorded for each infant included: - name, data and place of birth, gestational age, mode of delivery, Apgar score at 1 and 5 min, - need of resuscitation in delivery room: oxygen therapy and assisted ventilation, pH, PCO2 and base excess in cord blood, - respiratory distress syndrome (RDS) and surfactant therapy, - mean daily blood cuff blood pressure values, - type and duration of assisted ventilation, - oxygen dependency at 28 days and chronic lung disease (CLD) diagnosed on typical radiological findings, - intraventricular hemorrhage, diagnosed by serial ultrasound scans of the brain, - necrotizing enterocolitis (NEC) diagnosed when typical clinical and radiological findings were present, - patent ductus arteriosus (PDA) diagnosed by typical echocardiographic findings, - number, volume and date of packed red cells transfusions; treatment with erythropoietin.

Heparinized blood samples were obtained from the umbilical vein immediately after cord clamping at delivery and from a peripheral vein on days 7, 14 and 21.

Blood was immediately centrifuged, and all analyses were performed in plasma within 2 h of blood sampling to avoid the effects of storage. After centrifuging, the plasma and buffy coat were removed.

AOPP were measured using spectrophotometry on a microplate reader. AOPP concentrations were

Table 1. Clinical characteristics of the patients

Newborns (n°)	**72**
Sex	38 males – 34 females
Gestational age at birth (wks)	27.0 +/- 1.4 (24-29)
Neonatal birth weight (g)	856.86 +/- 183.55 (460-1240)
Mode of delivery: • Vaginal delivery • Elective cesarean section • Emergency cesarean section	 14 (19.4%) 10 (13.8%) 48 (66.6%)
Small for gestational age	16 (22.2%)
RDS	58 (80.5%)
Intubated	48 (66.6%)
Mode of ventilation: • CPAP • sIMV • HFVO	 15 (20.8%) 32 (44.4%) 19 (26.3%)
Surfactant	46 (63.8%)
ROP (stage 1 - stage 2)	16 (22.2%)

Abbreviations: RDS: respiratory distress syndrome, CPAP: continuous positive airway pressure, sIMV: synchronized intermittent mandatory ventilation, HFVO: high-frequency oscillatory ventilation, ROP: retinopathy of prematurity

Table 2. Chronological blood gas analysis, blood preassure, pulse oxymeter and O_2 administration data

	Cord blood	**Day 7**	**Day 14**	**Day 21**
pH	7.28 (7.11-7.30)	7.32 (7.29-7.37)	7.32 (7.25-7.40)	7.33 (7.29-7.38)
PaO_2	24 (20-32.4)	48.1 (32.7-61.4)	46.2 (36.5-64.6)	45 (35.5-57.1)
BE	-4.9 (-11.6 - -1.5)	-2.5 (-3.9 - -0.4)	-3.2 (-8.2 - -1.1)	-1.4 (-5.8 - -1.2)
	First hour of life	Day 7	Day 14	Day 21
Sat O_2	92 (81-93)	90 (80-92)	91 (84-94)	89 (82-92)
Systolic BP	46 (35-65)	47 (42-54)	57 (48-62)	60 (45-64)
Diastolic BP	24 (14-41)	25 (20-30)	31 (22-39)	34 (23-40)
FiO2 max	33 (30-60)	30 (23-42)	29 (22-30)	25 (21-35)

Values are median and quartiles (in brackets) BE: base excess, BP: blood pressure

expressed as micromolar chloramine-T equivalents. TH production was measured with a d-ROMs Kit by Diacron Srl, Grosseto, Italy, (8). NPBI plasma levels were detected by HPLC using the method previously described (22). Protein carbonyls concentration was determined by enzyme-linked immunosorbent assay (ELISA) using the Zentech PC Test Kit (Zenith Technology, Dunedin, New Zealand), by the method of Buss *et al.* (23).

The diagnosis and classification of ROP was based on serial ophthalmoscope examinations performed using the indirect binocular ophthalmoscope according to the protocol suggested by the Italian ROP Study Group, that was based on the International Classification of ROP (24). The first ophthalmologic examination was performed at an average of 3 weeks of age. The fundus was examined under topical anaesthesia with the aid of an eyelid speculum and a scleral depressor. Mydriasis was achieved by instillation of tropicamide 1% at 10 and 30 min before examination and a mixture of tropicamide 1% and phenylephrine 2.5% shortly before the examination. In non-ROP cases the examination was repeated every week until vascularization of the retina was complete. If ROP changes were present the examinations were more frequent.

The frequencies of infants with the following diagnoses were determined: CLD, intraventricular hemorrage (IVH, all degrees), ROP, PDA, NEC.

The babies were subdivided in two groups: group I (babies without ROP) and group II (babies who developed ROP stage 1-2).

The data, expressed as, median and quartiles, were analyzed for statistically significant differences by the non parametric test for two independent or related samples (Mann-Whitney U, Wilcoxon and ANOVA tests), using SPSS release 6.0 statistical package (Dynamic Microsystems Inc., Silver Springs, MD, U.S.A.) Initially associations between potential risk factors and ROP were assessed using the chi square test or Fisher's exact test. Considering the multivariate structure of the data, a stepwise, forward, logistic regression analysis was performed to determine the most important predicting factors.

4. RESULTS

Out of the 72 infants, 16 (22.2%) developed ROP stage 1-2. At baseline time, no differences on pH and base excess were found between babies who developed ROP and babies without ROP (pH: 7.36 +/- 0,06 *vs* 7.32 +/- 0,08; base excess: -1.68 +/- 2.38, *vs* -2.55 +/- 4.18, respectively) (Table 2).

All infants with ROP stage 1 -2 showed complete regression of retinopathy. No severe form of ROP (stage 3 or 4) was observed. Twenty four infant received blood transfusions in the first week of life (10 ml /Kg of packed red blood cells) Nobody received rhu-erithropoietin. No ascertained sepsis was observed in the study period.

Infant with ROP (stage 1-2) had, compared with those without ROP, significantly lower birth weight ($p < 0.0001$), lower gestational age ($p < 0.0001$), lower diastolic and systolic blood preassure ($p = 0.0002$ and $p < 0.0001$),

Table 3. Continuous and binary factors in regression analysis

Variable	Group I (Babies without ROP) n = 56	Group II (Babies with ROP stage 1-2) n = 16	p-value
Gestational age (wks)	27.38 +/- 1.52	26.05 +/- 1.4	<0.0001
Birth weight (g)	923.43 +/- 159	745.52 +/- 115.40	<0.0001
Systolic blood pressure (mmHg)	55.36 +/- 10.50	45.80 +/- 9.0	<0.0001
Intubation	36 (64.2%)	14 (82.3%)	<0.0001
Mechanical ventilation (SIMV or HFVO)	33 (58.9%)	14 (82.3%)	<0.0001
CLD	14 (25%)	9 (52.9%)	<0.0001
Postnatal steroid administration	8 (14.2%)	8 (47%)	<0.0001
Diastolic blood pressure	30.10 +/- 8.97	22.91 +/- 8.04	0.0002
RDS	41 (73.2%)	14 (82.3%)	0.004
Surfactant administration	38 (67.8%)	9 (52.9%)	0.004
Blood transfusions	18 (32.1%)	8 (47%)	0.0013
Apgar score to 5 min (range)	3-10	6-8	0.0036
FiO_2 max	34.25 +/- 16.05	43.96 +/- 22.17	0.017
PDA	24 (42.8%)	9 (52.9%)	0.05

Abbreviations: sIMV: synchronized intermittent mandatory ventilation, HFVO: high-frequency oscillatory ventilation, CLD: chronic lung disease, RDS: respiratory distress syndrome, PDA: patent ductus arteriosus

Table 4. Chronological analysis of markers of oxidative stress

		Cord blood	Day 7	Day 14	Day 21	p^1
NPBI (microg/ml)	No ROP	5.21 (2.6-10.5)	1.49 (0 - 3.5)	3.35 (1.28-5.29)	1.34 (0-4.7)	0.039
	ROP	3.09 (3-18)	1.65 (0.15-6.25)	2.10 (0-7.11)	0 (0-2.5)	NS
TH (U.Carr/L)	No ROP	63 (37-132)	108 (55-150)	123 (90-157)	125 (99 -158)	0.034
	ROP	70.5 (56.7-90.7)	69 (35.5-144)	106 (74.5-165.7)	171 (103-205)	0.016
AOPP (micromol/L)	No ROP	46.7 (18.8-98.4)	23.9 (13.5-121)	35.1 (16.5 -75.1)	61.5 (21.9-99.8)	NS
	ROP	29.8 (16.4-76)	21.5 (15.3- 58.3)	44.4 (32.2 -56.8)	34.2 (25.2 -34.2)	NS
CO groups (nanomol/mgprotein)	No ROP	0.26 (0.1-0.71)	0.25 (0-0.67)	0.20 (0- 0.45)	0.21 (0-0.61)	NS
	ROP	0.19 (0-0.39)	0.21 (0-0.77)	0.48 (0.1-0.8)	0.31 (0-0.30)	NS

Data are expressed as median and quartiles (in brackets) 1p-value for OS markers measured at different ages (ANOVA test) NS= not significant. Abbreviations: NPBI: non protein bound iron, TH: total hydroperoxides, AOPP: advanced oxidative protein products, CO: carbonyl groups

higher FiO_2 needed for resuscitation at birth (80% *vs* 30%, p = 0.02) Need of mechanical ventilation and steroid treatment for lung disease were significantly associated with ROP (stage 1-2) ($p < 0.0001$ and $p = 0.0016$, respectively) A significantly higher frequency of CLD (75% *vs* 28%, p = 0.04) was observed in infants with ROP (stage 1-2) than in those without ROP. The relationship of ROP to recorded factors are reported in Table 3. The variables on univariate analysis that gave a high correlation with the development of ROP were included in a multivariate step-wise logistic regression analysis. Among the variables included, the following factors had a significant predictive value for the development of ROP stage 1-2: birth weight (OR=0.95, 95% CI of OR =0.89-0.99; p =0.03), and O2 administration in delivery room (OR=0.90, 95% CI of OR=0.83-0.97; p =0.01).

The entity of OS was similar in all babies. No difference in markers of OS was observed between babies with ROP (stage 1-2) and babies without ROP (Table 4) NPBI significantly decreased in babies without ROP from time 0 to time 21 (p= 0.039) TH significantly increased from birth to 21 days both in babies with ROP (stage 1-2) and in babies without ROP (p= 0,034 and p= 0.016, respectively).

5. DISCUSSION

At birth, the newborn is exposed to a relatively hyperoxic environment caused by an increased oxygen bioavailability, with greatly enhanced generation of FR. In the preterm baby, the perinatal transition is accompanied by the immaturity of the antioxidant systems and the reduced ability to induce efficient homeostatic mechanisms designed to control cell-damaging FR. In the brain of infant rodents short exposures to nonphysiologic oxygen levels can trigger apoptotic neurodegeneration (25). We recently reported that this OS is able to induce renal damage and impairment in preterm newborns (11). The possible contribution of lipid peroxidation reaction in developing ROP has been demonstrated only in experimental animal models (26,27). We previously found that an OS occurr at birth and persists over the first week of life both in preterm and term babies (8-10). We suggested the need to use antioxidant strategies to minimize OS. No data exist on measurement of OS markers in newborns at high risk for ROP.

In this study we measured NPBI as a reliable index of FR production and TH, AOPP and CO groups as markers of lipid and protein oxidation in preterm babies in the first 3 weeks of life, with the aim to minimize OS and reducing the incidence and the severity of ROP.

We did not observe severe form of ROP (stage 3-4), 16 cases (22.2%) developed ROP stage 1-2.

The entity of OS was similar in all babies. NPBI significantly decreased in the first 3 weeks of life, reducing, in all population, the risk of FR generation as a consequence of Fenton reaction. In contrast, TH increased from birth to 21 days both in babies with ROP (stage 1-2) and in babies without ROP, suggesting that all preterm infants are physiologically prone to OS at birth because the

extrauterine enviroment is richer in oxygen than intrauterine enviroment. This hyperoxic challenge is accompanied by the immaturity of the antioxidant system and the reduced ability to induce antioxidant enzymes.

Table 2 shows variables significantly related to the occurrence of ROP stage 1 or 2. When these variables were entered into a logistic regression, birth weight and O_2 needed for resuscitation in delivery room were the factors having a significant predictive value for the development of ROP.

These data clearly highlight the role of oxygen as one of the main factors responsible of abnormal retinal development in very low birth weight infants. The role of oxygen is intriguing as a factor of retinal damage. Oxygen plays a different role for the retinal vasculature development according to the natural course of the disease, having direct effect on gene regulation and important toxicity from FR. ROP involves the cessation of normal angiogenesis (first phase) and subsequent hyperproliferative neovascular response to retinal ischemia (second phase) (28). The first phase is caused by early exposure of immature retina to hyperoxia, which down regulates vascular endothelial growth factor (VEGF) production and inhibits normal retinal vascularization (29). The proteins that control blood vessel formation have redox sensitive metallocenters that are sensors of oxygen tension. In this phase oxygen is deeply toxic. In the second phase oxygen is useful and must be delivered.

Oxygen administration in the delivery room for resuscitation determines hyperoxic challenge and predispose to ROP (20). It is likely that low oxygen early in life allows sequential and orderly development of retinal vessels and retina that decreases the area of avascular retina as the babies approaches 32 weeks of life, the average time during which ROP begins to develop. Moreover, minimizing hyperoxic challenge brings down OS one of the main mechanisms that injure the infant's vessels, thus lowering the risk of severe ROP.

This may be achieved by a tight protocol for oxygen administration in the immediate postnatal life likely contributing to prevent the early vasoobliterative phase due to hyperoxia-induced down-regulation of VEGF production. (19, 30, 31).

The data presented here report for the first time biochemical markers of lipid and protein peroxidation in very low birth weigth infants at high risk for developing ROP. The results show a low FR generation and damage in preterm babies managed with a strict control of oxygen administration. Because OS also occur in babies resuscitated with room air richer in oxygen than intrauterine environment, oxygen in delivery room must be used carefully to reduce its toxic effects. These data are relevant for neonatologists and contribute to guide management policies that are useful in avoiding, or at least minimizing, the risk of severe stages of ROP.

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Abbreviations: AOPP: advanced oxidative protein products, CO: carbonyl groups, CLD: chronic lung disease, FR: free radicals, IVH: intraventricular hemorrage, NEC: necrotizing enterocolitis, NPBI: non protein bound iron, OS: oxidative stress, PDA: patent ductus arteriosus, ROP: retinopathy of prematurity, TH: Total hydroperoxides

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