

Antioxidant Capacity and Oxygen Radical Diseases in the Preterm Newborn

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Background: Bronchopulmonary dysplasia, intraventricular hemorrhage, necrotizing enterocolitis, and retinopathy of prematurity may be different manifestations of oxygen radical diseases of prematurity (ORDP).

Objective: To test the hypothesis that the antioxidant capacity of cord blood serum will predict risk of ORDP.

Design: An inception cohort of premature neonates was followed up from birth until discharge or death to determine if outcome was related to cord blood serum antioxidant capacity, as determined by a manual assay measuring the relative inhibition of oxidation of 2,2'-azino-di-(3-ethylbenzthiazoline)-6 sulfonic acid (ABTS). Possible correlations between antioxidant capacity and various perinatal factors were also tested.

Setting: Level 3 newborn intensive care unit.

Patients: All inborn very low-birth-weight neonates from whom cord blood was available and for whom maternal consent was obtained were included. Newborns who died in the first week of life or who had major congenital malformations were excluded. A convenience sample of new-

borns weighing more than 1500 g was used to perfect assay and explore confounders.

Main Outcome Measures: Significant ORDP was defined as the presence of intraventricular hemorrhage greater than grade 2, retinopathy of prematurity greater than stage 1, bronchopulmonary dysplasia at the post-conceptual age of 36 weeks, or necrotizing enterocolitis with the hypothesis that neonates with ORDP will have lower antioxidant capacity in cord blood serum.

Results: Serum antioxidant capacity at birth correlated with gestational age for the entire sample of 41 neonates and for the 26 neonates born before 32 weeks' gestation. After correction for gestational age, cord serum antioxidant capacity did not correlate with maternal smoking, preeclampsia, chorioamnionitis, cord pH Apgar scores, or any of the ORDP studied.

Conclusion: Cord serum antioxidant capacity correlates with gestational age but does not predict ORDP risk.

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THE SURVIVAL rate of very low-birth-weight infants has greatly increased during the last 2 decades. However, bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), intraventricular-periventricular hemorrhage (IVH), and necrotizing enterocolitis continue to be important problems for these infants. Although each of these 4 disorders has a complex and poorly understood pathogenesis, in addition to prematurity, reactive oxygen species have been suggested as playing crucial roles.¹ In the presence of hypoxia-ischemia, hypoxanthine is generated from the breakdown of adenosine monophosphate. With reperfusion in the presence of oxygen, hypoxanthine is oxidized to uric acid with the generation of superoxide,

which can react with hydrogen peroxide in the presence of iron in the Haber-Weiss reaction to produce the highly reactive hydroxyl radical. Hydroxyl radical can damage DNA, cause lipid peroxidation, and damage disulfide bonds of proteins. Another important source of oxygen and nitrogen radicals is activated inflammatory cells. Hypoxia-ischemia and reperfusion injury may be important in the pathogenesis of IVH,² ROP,³ and necrotizing enterocolitis.⁴ High inspired oxygen concentration and activated inflammatory cells may be important in causing BPD.⁵

Antioxidant defense mechanisms of the body include cellular and extracellular enzymes (eg, superoxide dismutase, catalase, glutathione reductase, and peroxidase) and free radical quenchers (eg,

PATIENTS AND METHODS

This study was approved and monitored by the Committee for the Protection of Human Subjects in Research of St Peter's University Hospital, New Brunswick, NJ. Neonates born at St Peter's University Hospital between May 1, 1998, and May 1, 1999, were studied. Healthy neonates with birth weights of more than 1500 g were selected at random from mothers delivering in this period without any complications of pregnancy and delivery. Consent was sought from all mothers delivered of infants with birth weights less than 1501 g. Subjects were included in the study if maternal consent was granted and cord blood was available. Neonates who died in the first week of life or neonates with major congenital anomalies were excluded from the study.

Cord blood was collected in plain glass tubes, allowed to clot, and centrifuged. Serum that was visually free of hemoglobin was pipetted into plain plastic tubes that were capped and frozen at -25°C for up to 1 month before assay. Charts were reviewed for data pertaining to pregnancy, labor, delivery, neonatal resuscitation, gestational age, birth weight, and outcome. Gestational age was based on available menstrual history, first trimester ultrasound findings, and clinical examination results.

Antioxidant capacity was measured using the method described by Miller et al.⁶ Metmyoglobin and 2,2'-azino-di-(3-ethylbenzothiazoline)-6 sulfonic acid (ABTS) in powder form were dissolved in 5-mmol/L phosphate-buffered saline, pH 7.4, for final reaction concentrations of 6.1- $\mu\text{mol/L}$ metmyoglobin and 610- $\mu\text{mol/L}$ ABTS. Doubly deionized water or a known concentration of Trolox or an aliquot of the cord blood serum were added to the metmyoglobin-ABTS solution in a cuvette and mixed by serial inversions; absorbance at 600 μm was recorded. Then hydrogen peroxide to a reaction concentration of 250 $\mu\text{mol/L}$ was added to the cuvette, mixing was again accomplished

by serial inversions, and the cuvette incubated in a 37°C water bath for 3 minutes. The second reading was taken exactly 3.5 minutes after addition of the hydrogen peroxide substrate. Total antioxidant status was calculated by comparing inhibition by the serum specimen to inhibition by Trolox, with results expressed as Trolox equivalent antioxidant capacity (TEAC).

Intraventricular-periventricular hemorrhage was diagnosed from cranial sonograms performed in the first weeks of life and classified according to a modified classification of Papile et al.⁷ Since many small preterm infants have small IVHs of no clinical significance, we only considered grade 3 or 4 IVH as possibly representing hypoxic-ischemic reperfusion injury. Bronchopulmonary dysplasia was diagnosed by persistent pulmonary infiltrates and supplemental oxygen requirement at postconceptional age of 36 weeks. Retinopathy of prematurity was diagnosed by retina specialists and classified according to the international classification.⁸ Because many preterm infants have mild ROP that subsequently regresses, only disease of grade 2 or higher was considered significant. Necrotizing enterocolitis was diagnosed by a combination of clinical signs of feeding intolerance, abdominal distention, blood in the stool, and either pneumatosis intestinalis or intestinal perforation on abdominal radiographs and pathologic diagnosis after surgery. All these outcome measures were assessed by personnel unaware of the antioxidant status of the infants.

Data are presented as mean \pm SD. Categorical variables were analyzed using χ^2 test or the Fisher exact test as appropriate. Relationship of gestational age and TEAC level was assessed using linear regression. To control for gestational age, this relationship was used as a covariate in the analysis of variance to analyze the difference in TEAC levels in relation to various oxygen radical diseases. For continuous dependent variables, we used stepwise multiple regression with gestational age as the first variable in the equation. $P < .05$ was considered significant.

glutathione, vitamins C and E, and carotenoids in addition to serum albumin and metabolic products such as uric acid and bilirubin). In humans and most of the animals studied, these antioxidant enzymes are expressed in high concentration only at the end of gestation in preparation for the relatively hyperoxic extrauterine environment. Thus, preterm infants are vulnerable to oxidant stress. Cord blood antioxidant capacity is the result of overall intrauterine experience. Genetic variability, maternal oxidant stress, and maternal antioxidant capacity are likely to alter the cord blood antioxidant capacity. Total plasma antioxidant capacity along with cellular antioxidant enzymes at birth will determine how the infant will withstand the stress of being born into a relatively hyperoxic environment compared with the intrauterine experience. After birth, increased oxidant stress will result in a decrease in antioxidant capacity. Once the antioxidant capacity is overwhelmed, then an increase in oxidation products of lipids, protein, and nucleic acids is expected, resulting in tissue damage. Clinical manifestations of oxygen radical diseases depend on a balance between tissue damage and repair. Thus, a low antioxidant capacity of the body can predispose to the development of oxygen radical diseases. However, in pre-

term infants who have low levels of antioxidant enzymes, even normal levels of plasma antioxidant capacity may not prevent the development of oxygen radical diseases if the oxidant stress is severe enough.

Cellular levels of various antioxidant enzymes are difficult to measure; however, the antioxidant capacity of the extracellular fluid as reflected in the blood is relatively easy to measure. We used the assay developed by Miller and coworkers⁶ to measure the total antioxidant capacity of the cord blood. Our aim was to assess the relationship between cord blood antioxidant capacity and gestational age and to examine whether infants with oxygen radical diseases had lower total antioxidant capacity than infants without these disorders.

RESULTS

We measured the correlation of gestational age and TEAC levels in 41 newborns with birth weight of 1515 ± 887 g, gestational age of 31 ± 5 weeks, 1-minute Apgar score of 7 ± 2 , and 5-minute Apgar score of 8 ± 1 . There was a strong positive statistically significant correlation between cord serum TEAC level and gestational age, with an r value of 0.64 (**Figure 1**). Since all our infants with

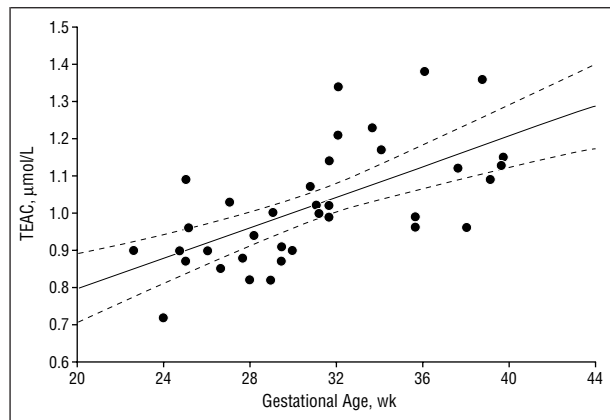


Figure 1. Relationship of gestational age and Trolox equivalent antioxidant capacity (TEAC) levels ($r=0.64$).

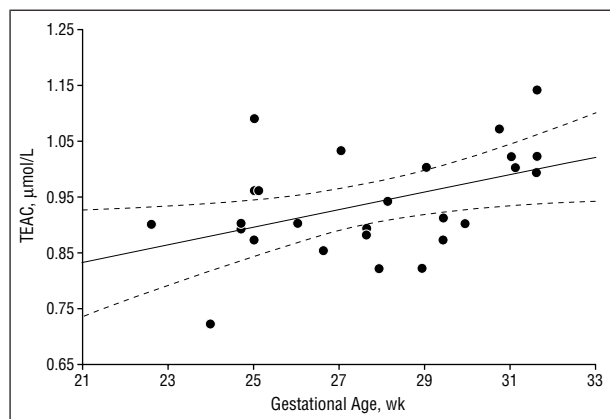


Figure 2. Relationship of gestational age and Trolox equivalent antioxidant capacity (TEAC) levels in neonates less than 32 weeks' gestational age ($r=0.45$).

oxygen radical diseases were born at a gestational age less than 32 weeks, we also looked at the correlation of gestational age and TEAC level in this subgroup of newborns (**Figure 2**). In this group, cord serum TEAC level was also positively correlated with gestational age ($r=0.45$, $P<.05$). Consequently, analysis of TEAC levels with regard to oxygen radical diseases was controlled for gestational age. Cord serum TEAC levels did not correlate with gestational age older than 32 weeks.

In infants less than 32 weeks' gestation, 12 had 1 or more oxygen radical diseases (IVH higher than grade 2, BPD at postconceptional age of 36 weeks, ROP greater than stage 1, and necrotizing enterocolitis), and 11 had none and served as controls. There was one death from overwhelming necrotizing enterocolitis at 1 month in the oxygen radical disease group and none in the control group. Some of the prenatal and postnatal characteristics of this group of newborns are shown in **Table 1**. As expected, the group with oxygen radical diseases had lower birth weight, gestational age, and 5-minute Apgar score and required more frequent supplemental oxygen and positive pressure ventilation in the delivery room. These newborns also had a higher incidence of respiratory distress syndrome. The number of male infants and black infants was not significantly different in the 2 groups. Similarly, antenatal steroid use, pathologic diagnosis of

Table 1. Prenatal and Postnatal Characteristics of Newborns With and Without Oxygen Radical Diseases of Prematurity (ORDP)

Characteristic	ORDP Present (n = 12)	ORDP Absent (n = 11)
Birth weight, mean \pm SD, g	735 \pm 202	1160 \pm 187*
Gestational age, mean \pm SD, wk	25.4 \pm 1.5	28.9 \pm 1.2*
Male, No. (%)	8 (67)	5 (45)
Black, No. (%)	4 (33)	8 (73)
Prenatal betamethasone, No. (%)	9 (75)	9 (82)
Chorioamnionitis, No. (%)	2 (17)	3 (27)
Preeclampsia, No. (%)	3 (25)	3 (27)
Smoking, No. (%)	5 (42)	2 (18)
Apgar score, mean \pm SD		
1 min	5 \pm 2	7 \pm 2
5 min	7 \pm 1	9 \pm 1*
Required oxygen in delivery room, No. (%)	12 (100)	6 (55)
Required positive pressure ventilation in delivery room, No. (%)	11 (92)	2 (18)
Respiratory distress syndrome, No. (%)	12 (100)	6 (55)

*Significantly different at $P<.05$.

Table 2. Trolox Equivalent Antioxidant Capacity (TEAC) Levels in Relation to Oxygen Radical Diseases of Prematurity (ORDP) in Newborns Less Than 32 Weeks' Gestational Age*

Manifestion	Mean \pm SD TEAC, μ mol/L	
	ORDP Present	ORDP Absent
Intraventricular hemorrhage > grade 2	0.87 \pm 0.10 (n = 4)	0.95 \pm 0.09
Bronchopulmonary dysplasia at postconceptional age of 36 wk	0.92 \pm 0.12 (n = 7)	0.92 \pm 0.07
Retinopathy of prematurity > grade 1	0.89 \pm 0.15 (n = 4)	0.92 \pm 0.07
Any oxygen radical disease	0.92 \pm 0.09 (n = 12)	0.93 \pm 0.08

*Twelve newborns had 1 or more oxygen radical diseases, and 11 newborns had none.

chorioamnionitis, and maternal smoking were not different between the 2 groups.

The cord blood serum TEAC levels are shown in **Table 2**. We analyzed these levels in relation to all IVH, IVH greater than grade 2, BPD at postconceptional age of 36 weeks, ROP greater than stage 1, and any 1 or more of these oxygen radical diseases. Although newborns with each of these diagnoses had lower mean cord blood TEAC levels, none of these differences were statistically significant.

We also analyzed the effect of cord blood pH, Apgar scores, preeclampsia, chorioamnionitis, and maternal smoking on cord blood TEAC levels. After adjustment for gestational age, no significant differences were found for any of these variables on cord blood TEAC levels.

COMMENT

Our results indicate a strong positive correlation of the serum antioxidant levels with gestational age. These results are consistent with those of Miller et al,⁶ who showed

a strong positive correlation between antioxidant capacity of cord blood plasma and birth weight. We also showed a positive correlation with gestational age in neonates less than 32 weeks' gestation. This is in contrast to a recent report of the antioxidant capacity of plasma from neonates born before 32 weeks' gestation,⁹ which did not show a significant correlation with gestational age. However, Miller et al obtained blood specimens in the first 12 hours of life and not from the umbilical cord. Bilirubin levels rise in the first few hours of extrauterine life and contribute significantly to antioxidant capacity.^{10,11} Sullivan and Newton,¹² using an assay that measures the ability of biologic specimens to inhibit auto-oxidation of bovine brain homogenate, reported a positive correlation between the antioxidant capacity of cord blood serum and gestational age. Similarly, Silvers et al,¹³ using a similar assay to measure the antioxidant capacity of cord blood plasma, found a positive correlation with gestational age. On the other hand, antioxidant capacity as measured by the total radical trapping capacity (TRAP) assay did not differ significantly between preterm and full-term infants.¹⁴ Biomarkers of reactive oxygen species damage have shown a negative correlation with gestational age. Thus, higher levels of the lipid peroxidation products malondialdehyde (MDA)^{15,16} and exhaled alkanes^{17,18} were found in preterm infants. Similarly, higher levels of carbonylated proteins were found in preterm infants.^{19,20}

Biomarkers of reactive oxygen species have been studied in preterm infants in relation to possible oxygen radical diseases. Schlenzig et al¹⁵ measured urinary MDA in 3-hour collections scattered throughout the first 30 days of extrauterine life from 45 preterm infants (25-35 weeks' gestation). The MDA excretion was higher in infants who developed BPD. Inder et al¹⁶ measured plasma MDA from 61 infants (birth weight <1500 g or gestational age <32 weeks) and compared 3 groups: infants who died, infants who survived but had BPD at postconceptional age of 36 weeks, and infants who survived free of BPD. Plasma MDA levels from cord blood and blood drawn on day 2 were similar among the groups, but on day 7 the infants who survived without BPD at 28 days had significantly lower levels of plasma MDA. Infants who died or developed BPD at 36 weeks had significantly higher day 7 plasma MDA levels than those who survived free of BPD. These differences remained significant after correction for gestational age. They also found that infants who developed ROP greater than stage 1 also had higher day 7 plasma MDA levels, but infants with IVH greater than grade 2 did not.

Pitkänen et al²¹ measured exhaled pentane and ethane daily for the first 5 postnatal days from 19 very low-birth-weight infants with respiratory distress syndrome. Eight infants with "good outcome" were extubated by 7 days and survived without BPD, ROP, or neurologic abnormality at 6 to 12 months of age. Eleven had "poor outcome," including 5 who died of respiratory failure or IVH and 6 survivors with BPD of whom 3 had ROP. Both maximal ethane and maximal pentane levels were higher in the poor outcome group. Varsila et al¹⁷ measured exhaled alkanes from 27 infants born before 32 weeks' gestation who required intuba-

tion for inadequate spontaneous ventilation. High alkane excretion was associated with death or BPD but not with IVH. Nycyk et al¹⁸ measured pentane exhalation daily for the first 7 postnatal days from 57 infants born at less than 33 weeks' gestation. Peak pentane exhalation was significantly associated with IVH, severity of IVH, mortality, and ROP but not with BPD at the postconceptional age of 36 weeks.

Carbonylation of protein in tracheal aspirates from day 3 has been found to be increased in infants who later developed BPD and was a stronger predictor of BPD than gestational age or fraction of inspired oxygen.¹⁹ At 24 to 48 hours after birth, neonates who subsequently developed BPD at 28 days of age also had higher plasma levels of allantoin (produced by the nonenzymatic oxidation of uric acid by reactive oxygen species) and higher ratios of allantoin to urate than neonates who were free of BPD.²² Schrod et al²³ found decreased ratios of uric acid to MDA in tracheal aspirates at 3 to 14 days from very low-birth-weight neonates who developed BPD at the postconceptional age of 36 weeks compared with very low-birth-weight neonates who required ventilatory assistance and were free of BPD.

Antioxidant capacity of blood has shown a more variable result in relation to the development of oxygen radical diseases of the preterm infant. Moison et al²⁴ found that plasma TRAP on day 10 was lower in neonates who developed BPD at 28 days of life. Silvers et al¹³ found lower plasma antioxidant capacity in the first 2 hours of life in infants who died, but among survivors the 2-hour plasma antioxidant capacity was not related to BPD at the postconceptional age of 36 weeks. Drury et al¹⁰ found lower levels of plasma antioxidant activity during the first 12 hours after birth in infants who subsequently developed BPD at 28 days of life. However, they found no relationship between plasma antioxidant capacity and IVH, ROP, BPD at postconceptional age of 36 weeks, or death. We also failed to correlate cord blood serum antioxidant levels with any of the oxygen radical diseases that we studied. This failure to correlate serum antioxidant levels with oxygen radical diseases may be due to the fact that extracellular antioxidant capacity is only one aspect of the total antioxidant capacity of the tissues. Alternatively, serial measurements of antioxidant capacity may correlate better with the development of oxygen radical diseases than 1 or 2 measurements at birth.

In conclusion, we found a strong positive correlation of cord blood serum antioxidant capacity with gestational age. However, we were unable to correlate cord blood serum antioxidant capacity with the development of any of the oxygen radical diseases that we studied.

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