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Plasma Chain-breaking Antioxidants in Preterm Infants with Good and Poor Short-term Outcome

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Many complications of prematurity have been suggested to result from free radical generation and an inadequacy of antioxidative capacity. We measured the plasma total peroxyl radical-trapping capability (TRAP) and concentrations of the main chain-breaking antioxidants contributing to it, i.e. uric acid, ascorbic acid, α-tocopherol, protein sulfhydryl groups and bilirubin, in 21 preterm infants with a mean birth weight of 1440 g and gestational age of 30 wk. The infants were divided into two groups according to their short-term outcome; the good outcome group (GOG) ($N = 11$) with no signs of morbidity and the poor outcome group (POG) ($N = 10$) with intraventricular haemorrhage and/or bronchopulmonary dysplasia and/or retinopathy. Arterial blood samples were obtained 3 and 10 days postpartum. TRAP was measured with a chemiluminescent method. As a comparison, venous blood samples from 13 adults (aged from 18 to 34) were used. At day 3 the poor outcome group had significantly higher TRAP than the good outcome or control group, mainly because of elevated uric acid concentration. Also the concentration of unidentified antioxidants was significantly lower in GOG. By day 10 the TRAP decreased substantially in both groups. However, from the components of TRAP, both ascorbate and the unidentified fraction decreased more in POG ($p = 0.017$ and 0.021, respectively). Furthermore in POG on day 10 urate concentration did not significantly differ from day 3 values. In conclusion, in preterm infants high TRAP was associated with high plasma uric acid concentration and a poor short-term prognosis.

Keywords: Ascorbic acid, free radicals, α-tocopherol, uric acid

INTRODUCTION

Oxygen-related free radicals have been suggested to be involved in the development of several complications of prematurity, e.g. bronchopulmonary dysplasia, retinopathy of prematurity, necrotizing enterocolitis, ischemic brain damage and intraventricular haemorrhage.[1–5] On the basis of animal experiments the antioxidant

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defences are proposed to be insufficient in preterm infants.[6] Studies on human infants have given conflicting results; Autor *et al.*[7] reported the activity of pulmonary superoxide dismutase to increase as gestation progressed, while McElroy *et al.*[8] found the activity of both pulmonary superoxide dismutase and glutathione peroxidase to remain constant throughout gestation and that of catalase to increase towards term gestation.

In plasma, transferrin and ceruloplasmin act as preventive antioxidants inhibiting hydroxyl radical production by binding and oxidizing iron.[9] Uric acid,[10] ascorbic acid,[11] α-tocopherol,[12] protein sulfhydryl groups (SH),[13] and in neonates also bilirubin,[14,15] are responsible for the removal of reactive oxygen species from plasma and form most of the plasma total peroxyl radical-trapping capability (TRAP). The TRAP in cord blood of preterm infants has been found to be as high as in term infants, and even higher than in adults.[16]

We measured TRAP and the plasma concentrations of the major chain-breaking antioxidants successively 3 and 10 days postpartum in 21 preterm infants to study whether their short-term outcome is related to their capability to extinguish oxygen radicals.

METHODS

Patients

Twenty-one infants successively, admitted to the neonatal intensive care unit of Tampere University Hospital and needing supplementary oxygen for at least 48 h, were enrolled in the study. The infants were divided into two groups according to their short-term outcome; the good outcome group (GOG) consisted of 11 infants who were neurologically normal at discharge, had a normal cranial ultrasonography and showed no evidence of bronchopulmonary dysplasia or retinopathy of prematurity. The poor outcome group (POG) consisted of 10 infants, of whom 5 had intraventricular haemorrhage grade III or IV (3 with hydrocephalus and 1 with cystic lesions), 4 died in the neonatal period (one of respiratory distress syndrome and sepsis and 3 of intraventricular haemorrhage and respiratory distress syndrome) and 1 had bronchopulmonary dysplasia and retinopathy of prematurity. The occurrence of intraventricular haemorrhage was verified with cranial ultrasonography.[17]

Clinical characteristics of the groups are presented in Table I. Gestational age was determined

TABLE I Clinical characteristics of the infants with good or poor short-term outcome

		Good outcome ($N = 11$)	Poor outcome ($N = 10$)
Gestational age (wk)[§]		31 (29–33)	30 (28–32)
Birth weight (g)[§]		1633 (1249–2018)	1231 (919–1543)
Apgar score at 5 min*		7 (4–9)	4 (1–8)
Number of infants who needed			
supplementary oxygen	at day 3	10	10
	at day 10	7	8
mechanical ventilation	at day 3	10	10
	at day 10	3	6
vasoactive drugs	at day 3	2	6
	at day 10	0	0
indomethacin	at day 3	2	0
	at day 10	3	4
diuretics	at day 3	0	4
	at day 10	2	4

[§] Mean (95% CI).
* Median (range).

by menstrual history and/or ultrasonography performed before 20 weeks of gestation. Three mothers in the good and 2 mothers in the poor outcome group had preecplampsia. Infants in the poor outcome group had lower median Apgar score at 5 min and needed a higher mean fractional inspiratory oxygen tension during the first 3 days than those in the good outcome group (60% [95% CI 47–74] and 39% [33–46], respectively, $p = 0.016$). All infants received antibiotics during the first days. The need of vasoactive drugs was higher in the poor than in the good outcome group. Enteral feeding was started at a mean age of 1.8 and 5.5 days in the groups with good and poor outcome, respectively. Three infants in the poor outcome group were without enteral feeding during the study period. Eighteen infants received parenteral nutrition with intravenous lipid emulsion (20% intralipid *R*) (8 and 10 in the good and poor outcome groups, respectively). None of the infants received supplementary ascorbic acid or α-tocopherol during the first 10 days. A control group consisted of 13 healthy volunteers (8 females and 5 males) aged from 18 to 34 years (26.8 ± 1.6 y).

Blood Sampling

Blood samples were drawn from the umbilical or radial artery at days 3 and 10 and from antecubial vein in the control group after a 4 h morning fast. The blood tests were taken on days 3 and 10 because the first days are the most critical periods in neonatal life and after 9 days the situation is more stabilized. Two infants in the poor outcome group died before the age of 10 days and the second blood sample was not available. In addition, the second plasma sample was lost for 1 infant in the poor outcome group. Blood samples, anticoagulated with EDTA, were shielded from light, cooled on ice and centrifuged within 20 min of withdrawal at 4°C 1500 rpm for 15 min. Plasma was separated and stored at −70°C for further analyses. An additional tube for ascorbate determination was prepared by adding to fresh plasma an equal volume of 5% meta-phosphoric acid containing iso-ascorbic acid as an internal standard. These tubes were also stored at −70°C.

Determination of TRAP

TRAP was measured with a chemiluminescent method.[17,18] Peroxyl radicals were produced at constant rate by thermal decomposition of ABAP [2,2-azo-bis(2-amidinopropane) hydrochloride]. Luminol chemiluminescence induced by peroxyl radicals was measured with a Pharmacia LKB Wallac Luminometer 1251. A PC measured the chemiluminescence at 35 s intervals, and after 15 min, when the chemiluminescence was stabilized, 20 ml aliquot of plasma was added into the cuvette and change in the chemiluminescence was monitored.

A water-soluble α-tocopherol analogue, TROLOX C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), known to trap 2 peroxyl radicals per 1 molecule (i.e. its stoichiometric factor is 2.0) was used as a standard.[18] The linear regression line for Trolox was designed and TRAP values were converted to mmoles of peroxyl radicals trapped by one liter of the sample. Both intra- and inter-assay variation of the method was 2% (CV, $N = 10$). Plasma TRAP was not changed within 2 months when the samples were stored at −70°C.

In addition to the direct measurement of TRAP, a calculated TRAP (TRAPcalc) was determined by using the plasma concentrations [mmol/l] of the individual antioxidants and their experimentally determined stoichiometric factors:[19] $\text{TRAPcalc} = 2.0 \times [\text{uric acid}] + 0.4 \times [\text{SH}] + 2.0 \times [\text{bilirubin}] + 0.7 \times [\text{ascorbic acid}] + 2.0 \times [\alpha\text{-tocopherol}]$. The stoichiometric factors indicate the molar amount of peroxyl radicals trapped by each mole of antioxidant. The difference between the measured TRAP and TRAPcalc is the unidentified fraction of the TRAP (UNID). Due to its high contribution, bilirubin was included only in TRAPcalc of neonatal samples.

Other Laboratory Determinations

The concentrations of uric acid and ascorbic acid were measured by HPLC with an electrochemical detector according to Frei *et al.*[11] and α-tocopherol was determined with a modified method of Catignani *et al.*[19] A detailed description of these methods and their modifications has been published earlier.[19] The SH groups were determined according to Ellman[20] and bilirubin by direct dual wavelength absorptiometry.

Statistical Analysis

The results are expressed as means $\pm$SEM. The statistical analysis was carried out by general linear models analysis of variance (GLM) and repeated measures ANOVA (SOLO Statistical System, BMDP Statistical Software, Inc., Los Angeles, CA, USA). Multiple regression analysis and Student's *t*-test were also calculated by the same software.

Ethics

The parents gave a written informed consent before their infant was enrolled in the study. The study protocol was approved by the Ethics Committee of Tampere University Hospital.

RESULTS

At day 3 TRAP, TRAPcalc and UNID were significantly higher in the poor outcome group (POG) compared to the good outcome group ($p < 0.0005$). The difference in TRAP between good outcome group (GOG) and POG at day 3 was 1091 µmol/l. In the components of TRAP, uric acid and UNID were significantly higher in POG (Table II). The bilirubin concentrations in GOG and POG were 93 ± 10.7 and 82.5 ± 9.5 µmol/l, respectively.

By day 10 a substantial decrease in TRAP was seen in both groups compared to day 3. From the identified antioxidants, urate, ascorbate and bilirubin were decreased from day 3 to day 10 in GOG, while only ascorbate and bilirubin decreased in POG. In both groups, tocopherol concentration in plasma increased. In repeated measures ANOVA analysis, unidentified antioxidants and ascorbate concentration in POG declined more than in GOG (Table II).

The percentage contributions of uric acid, ascorbic acid and α-tocopherol to the TRAP were similar in both groups, whereas SH and bilirubin accounted for a significantly higher proportion of the TRAP in the good compared to the poor outcome group (Table III). UNID accounted for a significantly higher share of the TRAP in the poor compared to the good outcome group.

In Spearman rank correlations between TRAP and the plasma concentrations of various antioxidants high uric acid clearly ($R = 0.988$, $p < 0.001$) correlated with poor outcome at day 3 (Table IV).

TRAP and the concentrations of the individual antioxidants showed no correlation with gestational age or birth weight. Apgar score at 5 min had a negative correlation with UNID in the poor outcome group at day 3 ($R = -0.631$, $p = 0.038$).

DISCUSSION

Plasma total antioxidative capacity was assessed by measuring the efficacy of a plasma sample to quench standardized peroxyl radical generation. A water-soluble α-tocopherol analogue, TROLOX, was used as standard. In addition, the contributions of individual plasma antioxidants to the TRAP were determined by using their known stoichiometric factors. It is controversial whether TRAP can be used to evaluate plasma antioxidant capacity.[21] However TRAP and its components are relatively easy to determine and the tests are reproducible.[17] As TRAP measures the total peroxyl radical-trapping capacity, practically all chain-breaking antioxidants are included while, e.g., the preventive antioxidants

TABLE II The measured, calculated and unidentified TRAP and the plasma concentrations of antioxidants to the TRAP. Results are presented as means (α-tocopherol, SH-groups and bilirubin) or geometric means (all TRAPs, uric acid and ascorbic acid) and 95% of confidence intervals (in parenthesis)

	Day	Good outcome ($N = 11$)		Poor outcome ($N = 10$)*		p-value‡
		Concentration	p-value†	Concentration	p-value†	
TRAP, measured, μmol/l	3	1096 (1023–1174)	0.009	2187 (1479–3236)	0.018	< 0.001
	10	852 (724–1000)		1445 (724–2818)		0.047
TRAP, calculated, μmol/l	3	912 (851–977)	0.004	1479 (1000–2188)	0.045	0.009
	10	708 (603–832)		1023 (512–2042)		n.s.
TRAP, unidentified, μmol/l	3	178 (135–229)	n.s.	629 (437–1096)	0.013	< 0.001
	10	151 (102–223)		389 (182–832)		0.012
Uric acid, μmol/l	3	229 (204–263)	< 0.001	490 (282–832)	0.030	0.005
	10	132 (107–166)		288 (109–741)		0.033
Ascorbic acid, μmol/l	3	20.4 (16.2–26.3)	< 0.001	41.7 (19.5–89.1)	< 0.001	n.s.
	10	7.2 (5.1–10.0)		8.9 (4.5–18.2)		n.s.
α-Tocopherol, μmol/l	3	5.2 (4.3–6.1)	< 0.001	5.4 (3.5–7.4)	0.006	n.s.
	10	12.6 (10.5–14.7)		12.7 (7.6–17.9)		n.s.
Protein SH-groups, μmol/l	3	579 (543-614)	n.s.	576 (518–635)	n.s.	n.s.
	10	628 (556–700)		553 (452–654)		n.s.
Bilirubin, μmol/l	3	93 (69–117)	0.044	82 (61–104)	n.s.	n.s.
	10	76 (54–98)		76 (31–121)		n.s.

n.s. = not significant.
* At day 10, $N = 7$.
† Difference between days 3 and 10 within groups, Student's two-tailed t-test for paired samples.
‡ Difference between groups, Student's two-tailed independent t-test.

TABLE III Percentage contribution of plasma antioxidants to the TRAP at the ages of 3 and 10 days in infants with good and poor short-term outcome: mean (95% confidence interval)

	Day	Good outcome ($N = 11$)		Poor outcome ($N = 10$)*		p-value‡
		% Contribution	p-value†	% Contribution	p-value†	
Uric acid	3	41.2 (37.3–46.5)	< 0.001	44.6 (38.0–51.2)	n.s.	n.s.
	10	32.0 (27.7–36.2)		41.1 (28.2–54.0)		n.s.
Ascorbic acid	3	0.8 (0.3–1.3)	0.026	1.2 (0.5–1.9)	0.004	n.s.
	10	0.2 (−0.1–0.4)		0.1 (−0.2–0.5)		n.s.
α-Tocopherol	3	0.8 (0.2–1.4)	0.002	0.2 (−0.1–0.6)	0.033	n.s.
	10	2.6 (1.8–3.4)		1.9 (0.1–3.6)		n.s.
Protein SH-groups	3	20.8 (18.7–22.9)	0.002	11.5 (7.2–15.8)	0.035	< 0.001
	10	29.0 (24.9–33.1)		17.7 (9.0–26.4)		0.006
Bilirubin	3	16.3 (12.2–20.3)	n.s.	7.9 (4.5–11.3)	n.s.	0.002
	10	16.8 (12.8–20.8)		8.9 (0.7–17.10)		0.035
TRAP unidentified	3	16.4 (12.7–20.0)	n.s.	31.7 (27.0–36.4)	0.009	< 0.001
	10	16.5 (9.9–23.2)		27.7 (21.5–33.9)		0.027

n.s. = not significant.
* At day 10, $N = 7$.
† Difference between days 3 and 10 within groups, Student's two-tailed t-test for paired samples.
‡ Difference between groups, Student's two-tailed independent t-test.

are excluded. However, the role of water-soluble peroxyl-radicals in triggering peroxidation processes *in vivo* is uncertain, and the capacity of a plasma sample to trap other not water-soluble biologically relevant radicals cannot be derived from its TRAP value. Also, as the efficacies of various antioxidants are different, TRAP does not reveal the quality of the antioxidative defence

TABLE IV Spearman rank correlation between the TRAP and the plasma concentration of various antioxidants in preterm infants with good or poor short-term outcome

	Day 3		Day 10	
	Good outcome $N=11$	Poor outcome $N=10$	Good outcome $N=11$	Poor outcome $N=7$
Uric acid	0.418	0.988***	0.815**	0.821*
SH	−0.009	−0.030	−0.682*	0.500
Bilirubin	0.027	−0.006	0.731*	0.643
Ascorbic acid	−0.036	0.830**	0.280	0.714
α-Tocopherol	−0.325	−0.571	0.092	−0.346

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

system. In spite of its limitations, TRAP may be useful in studying the body reactions to oxidative stress or other pathophysiological conditions.[22–24]

Our results showed that the plasma TRAP of preterm infants was comparable with or even higher than that found in adults.[25] Lindeman *et al.*[16] showed that in cord blood TRAP and TRAPcalc are higher in preterm than in term infants or in adults. Our own measurements of TRAP in cord plasma of 7 term infants (mean 972 mmol/l, 95% CI 915–1029, unpublished results) have given results comparable to those of Lindeman *et al.*[16] Sullivan[26] and Karmazsin[27] measured the capacity of a serum sample to prevent spontaneous auto-oxidation in bovine brain and reported the serum antioxidant capacity to be lower in preterm than in term infants both in cord sera and during the first days postpartum. The conflicting results may be explained by differences in methodology. The bovine brain spontaneous auto-oxidation method measures the preventive antioxidants in serum (e.g., ceruloplasmin and transferrin), while TRAP measures peroxyl radical quenching. In the study of Miller *et al.*[28] babies of less than 32 weeks gestation turned out to be antioxidant-deficient at birth. The material consisted of 16 babies, of whom 10 had good outcome. In that method based on the absorbance of the $ABTS^{\bullet+}$ radical cation the components are almost equal compared with TRAP (which measures plasma SH-groups instead of pure albumin) but the contribution of uric acid in TRAP is clearly higher (ca. 33% versus 43–52%). A similar correlation between TRAP and uric acid has also been shown by Lindeman *et al.*[16] In the study of Miller *et al.*[28] no difference was shown in the uric acid concentrations between the good and poor outcome babies or during the days of follow-up. In this study the uric acid concentration was significantly higher in the infants with poor outcome than in those with good outcome. In both groups a clear decrease was seen in day 10 and a similar decrease has been found in healthy preterm babies.[29]

The observed increase of plasma uric acid concentration in poor outcome group may have a dual explanation. The urinary excretion of uric acid is influenced by extra cellular volume and glomerular filtration.[30] The renal function was probably impaired at least in some of the infants in the poor outcome group, as 4 of them needed diuretics at day 3 compared to none in the good outcome group. However, as 9 infants out of 10 in the poor outcome group had higher TRAP than any infant in the good outcome group, also factors other than decreased urinary secretion of uric acid were possibly involved. Uric acid may also act as a reducing agent in the plasma, which contains non-protein bound iron. Plasma of preterm babies contains such iron[31,32] and has been found to stimulate peroxidation of pulmonary surfactant liposomes.[31] However, the reduction rate of Fe^{3+} by urate is very slow (0.02 nmol/min).[32]

Uric acid is also able to chelate iron in human plasma, thus preventing ascorbic acid from iron-catalyzed oxidation.[33]

However, the increase of uric acid concentration under oxidative stress could be interpreted as an inborn defence mechanism. Under hypoxia-ischemia, hypoxanthine is formed by breakdown of the adenine nucleotides ATP, ADP and AMP.[34] Furthermore, during the period of ischemia xanthine dehydrogenase is proteolytically converted to xanthine oxidase.[35] Under the following reperfusion period xanthine oxidase converts hypoxanthine to uric acid with a simultaneous production of oxygen-related free radicals.[36] On the other hand, uric acid is a potent chain-breaking antioxidant,[10] and in physiological concentrations, *in vitro*, it has also been shown to inhibit xanthine oxidase.[37] The more frequent use of vasoactive drugs in the poor outcome group suggests lability of blood pressure and a risk to ischemia-reperfusion and the subsequent conversion of xanthine dehydrogenase to oxidase. Free radical production with a possible damage to cell components may therefore have occurred more frequently in the infants with poor outcome than in those with good outcome.

The contribution of antioxidant vitamins to the TRAP was only marginal. In case of α-tocopherol it was 0.2–2.6%. α-tocopherol has been reported to prevent intraventricular haemorrhage by scavenging free radicals,[38] while studies on its ability to prevent retinopathy of prematurity have given conflicting results.[39,40] We did not find any differences in the plasma α-tocopherol concentrations between the groups with or without intraventricular haemorrhage. The number of infants studied may, however, have been too small for any conclusions on that. The increase of α-tocopherol concentration from day 3 to day 10 can be explained by postnatal lipid feeding and the concomitant increase of the plasma lipid concentration (mean serum cholesterol concentration rose from 2.05 to 2.65 mmol/l), and the subsequent redistribution of α-tocopherol between organs and plasma.

In experimental conditions ascorbic acid is a potent free radical scavenger in plasma and its therapeutic use has been proposed to be of benefit in the prevention of free radical mediated diseases.[41] Despite the high efficacy of ascorbic acid to quench free radicals, its contribution to the TRAP was only 0.1–1.2%. This is significantly less than in cord blood reported by Lindeman *et al.*[16] To ensure that decomposition during prolonged storage was not the reason for this, internal standards containing 5% meta-phosphoric acid and iso-ascorbic acid were prepared. However, the decline in ascorbic acid concentration after birth[29] may be beneficial in newborns with low ceruloplasmin concentration and nonprotein-bound iron. This decline may prevent ascorbic acid from acting as a prooxidant, reducing nonprotein bound ferric iron (Fe^{3+}) to dangerous ferrous (Fe^{2+}) form.[33,42] The higher concentrations obtained at birth[16] are supposed to protect against peroxidative damage when the baby moves from a low- to a high-oxygen environment.[29] The postnatal rise detected also in this material may be due to the maturation of antioxidant enzyme systems and renal tubular function.[43]

The percentage share of UNID was higher in the infants with poor outcome (27.7–31.7%) than in those with good outcome (16.4–16.5%). According to the study of Lindeman *et al.*[16] the UNID accounts for 22.5% of the TRAP in cord blood of preterm infants. This UNID must be a compound present in sufficient concentrations in plasma or a combination of several minor factors. According to our previous results, it is supposedly an endogenous product,[22–24] but antioxidants of a nutritional origin, like flavonoids or benzylisoquinone alkaloids, cannot be totally ruled out. The difference between these two studies is explained by bilirubin, which was not included in the TRAPcalc in Lindeman's study. Our first measurements were, however, made at the age of 3 days, when the infants may already have been exposed to ischemia-reperfusion, which may have influenced their UNID.

A possible bias caused by the differences in clinical characteristics between the groups has to be taken into account. The infants in the poor outcome group tended to be smaller and the mean Apgar score was also lower in that group. However, as TRAP did not correlate with either birth weight or gestational age, factors other than immaturity were also involved. Preeclampsia is associated with increased TRAP in the mother.[19] In this study, the prevalence of preeclampsia was similar in both groups. As the number of infants in the poor outcome group was small at day 10, the results must be interpreted with caution.

In conclusion, in preterm infants high TRAP was associated with a poor short-term prognosis. Increased TRAP was predominantly a consequence of high plasma uric acid concentration which accounted for over 40% of the TRAP.

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