Antioxidant activity, packed cell transfusions, and outcome in premature infants

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Abstract

***Aim*—To evaluate the relative importance of biochemical markers of antioxidant status, gestational age, and parameters of neonatal care in the clinical outcome of premature infants.**

***Method*—A prospective, observational, longitudinal study of the association between these factors was conducted. Blood was collected from an in situ arterial line within two hours of birth and at intervals thereafter, when blood was drawn for routine clinical purposes. Outcome was assessed as death, or survival with or without bronchopulmonary dysplasia (BPD). One hundred and forty four babies of 22 to 39 weeks of gestation, who required intensive care at the Jessop Hospital for Women, between January 1993 and April 1994, were recruited.**

***Results*—Low gestational age at birth was the most important predictor of mortality and the development of BPD. Having corrected for gestational age, low plasma antioxidant activity at birth was an independent risk factor for mortality. Plasma vitamin C at birth was significantly higher in the babies who died compared with those with a good outcome, but this effect was not sustained after correcting for gestational age. Repeated measures of Analysis of Variance revealed a postnatal increase in antioxidant activity, caeruloplasmin, retinol, cholesterol corrected α tocopherol, and red blood cell superoxide dismutase (SOD) activity. Vitamin C, on the other hand, declined in all groups after birth. Logistic regression analysis revealed that the greater the number of packed cell transfusions received during intensive care, and the higher the concentration of vitamin C on the second day of life, the greater the risk of developing BPD.**

***Conclusions*—After correcting for the effect of gestational age, low plasma antioxidant activity at birth was an independent risk factor for mortality. Frequent blood cell transfusions over the first week of life are associated with an increased risk of developing BPD. This association may be causal.**

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Several conditions, such as bronchopulmonary dysplasia (BPD), necrotising enterocolitis (NEC), and retinopathy of prematurity (ROP), are almost exclusive to infants who have received neonatal intensive care. These conditions make a substantial contribution to mortality and morbidity in the premature infant. Their aetiology is complex and probably multifactorial, but there is increasing experimental and clinical evidence to suggest the involvement of reactive oxygen species (ROS) in their pathogenesis

Many different factors may contribute to the aetiology of BPD, and it is essential that all such factors are taken into account. Much of the relevant data in this area have come from small studies, usually focusing on one or two factors of interest. In particular, there are few longitudinal data collected soon after birth. We have conducted a large longitudinal study in premature babies from birth until discharge to assess the relative importance of antioxidant variables at birth and postnatally, together with gestational age and parameters of neonatal intensive care, as determinants of mortality and the development of BPD.

Methods

One hundred and forty four babies were recruited at the Jessop Hospital for Women between January 1993 and April 1994; parental consent had been obtained. Infants were excluded from the study if they had any congenital malformations or inherited metabolic abnormalities (table 1). The study was approved by the Combined Sheffield Hospital Trust Ethics Committee.

A prospective, observational, longitudinal study was made of the association between biochemical markers of antioxidant status in blood, gestational age, and parameters of neonatal care and outcome. Outcome was assessed as death, or survival with or without BPD. BPD was defined as an oxygen requirement at 36 weeks of corrected age. Information was collected regarding the details of neonatal care, including hours of intermittent positive pressure ventilation (IPPV) and number of packed cell transfusions received. Antenatal use of steroids was also recorded. Whole blood (500 μl) was collected within two hours of birth from an in situ arterial line and, where possible, daily for the first four days of life and weekly thereafter at the time of routine clinical sampling, until discharge. Parental consent was given and ethics approval obtained for this additional blood sample to be taken. Blood samples were centrifuged immediately: plasma was stored at −70°C for the measurement of caeruloplasmin, vitamins A and E, cholesterol, and antioxidant activity. Aliquots were also stored at −70°C with metaphosphoric acid for the measurement of vitamin C. Red blood cells were washed and

Table 1 Characteristics of study group

Characteristics	**Values*	
Female	57	
Male	87	
Total	144	
Gestational age (weeks)		
Mean (SD)	28.9	(2.98)
Median	29.0	
Range	22.1–39.0	
Birthweight (g)		
Mean (SD)	1287	(552)
Median	1210	
Range	450–4250	
Oxygen therapy	140	
Ventilated	135	
Non-survivors	39	
Well survivors	80	
BPD survivors	25	

*Values are absolute numbers of subjects unless otherwise stated.

stored at −20°C for the measurement of superoxide dismutase activity.

Retinol and α tocopherol were extracted from plasma and measured by reverse phase isocratic high performance liquid chromatography.[1] In order to correct plasma α tocopherol concentrations for plasma lipid, the plasma cholesterol of each sample was measured using an enzymatic colorimetric assay[2] with a cholesterol PAP, uni kit II (Hoffman La Roche). Vitamin C was measured using an automated fluorometric technique.[3] The ability of plasma antioxidants to inhibit lipid peroxidation in a system to which no free radical initiator was added, was also assessed.[4] Antioxidant activity was expressed as the volume of plasma required for maximal inhibition of peroxidation and was termed Dmax. The higher the value for Dmax, the lower the antioxidant activity of the plasma. Plasma caeruloplasmin was measured turbidimetrically using a method automated for a centrifugal analyser with human caeruloplasmin kit. Superoxide dismutase activity was determined in haemolysates of red blood cells,[5] and expressed relative to the haemoglobin concentration of each haemolysate.[6]

STATISTICS

Details of each baby recruited, including information about outcome, and all biochemical results were stored in a Dataease database. The data were analysed using SPSS for Windows, excluding all babies with missing data for one or more variables in the model.

To determine which variables had most impact on mortality, a survival analysis was performed for non-survivors and survivors with or without BPD, using data collected at birth.[7] Total time in the study was calculated for each baby and entered into the analysis with the dependent and independent variables. This was total time alive in non-survivors and from birth until the time of the last sample in survivors with or without BPD. Gestational age, birthweight, use of antenatal steroids and all biochemical variables measured at birth were entered into the analysis as independent variables.

A Repeated Measures Analysis of Variance was carried out to identify postnatal changes in biochemical variables in the three outcome groups. Where postnatal changes were revealed these were explored further using a paired *t* test.

Logistic regression analysis was performed at birth and on the second, third, and eighth days of life for well survivors and survivors with BPD, to determine which variables were most important to the development of BPD. These days were chosen for analysis because the data were most complete. Gestational age, birthweight, the use of antenatal steroids and all biochemical variables measured at birth were entered into the birth analysis as independent variables. The use of antenatal steroids, the total number of packed cell transfusions, hours of IPPV and all biochemical variables measured on the appropriate days were entered into the second, third, and eighth day analyses as independent variables. As BPD was defined in terms of an oxygen requirement, the duration of oxygen supplementation was not included as an independent variable in this analysis.

A forward stepwise method (using conditional likelihood for inclusion of terms) was used for model selection, for both types of analysis. The variable with the largest positive or negative correlation with survival was considered for entry into the equation by forward selection if the level of significance obtained was less than or equal to 0.05. Once a variable was entered into the equation, the statistics for remaining variables were used to select the next most important one for entry into the equation, and so on.

After obtaining the results of the analysis the percentage relative risk for each unit of a variable selected was obtained by the following formula:

[exponential (β)-1.0] x 100 equation 1.

Similarly, the increase in risk of an event occurring in one baby, compared with any other baby, for a particular variable was calculated in the following way:

exponential [(*variable in baby one*)−(*variable in baby two*) × β coefficient]

Results

As the main aim of this paper was to present results of multiple regression analyses of the data, with quality of clinical outcome as the outcome variable, biochemical data will not be discussed in detail. A summary of the results of the biochemical analyses of blood samples collected at birth, and packed cell transfusions within 12 hours of birth, is presented in table 2. For Dmax and caeruloplasmin concentration, and number of packed cell transfusions, data were not normally distributed and are therefore presented as a median and range. On the day of birth, plasma vitamin C and retinol were significantly lower and plasma antioxidant activity was significantly higher in babies with a good outcome than in those who died.

Biochemical data from samples collected at different postnatal ages are given in table 3. Data are included only for those babies who remained in the study for eight days. Babies with missing data, and those who died or who were discharged during this period, are not included. Repeated Measures Analysis of Variance revealed a postnatal increase in antioxidant activity, caeruloplasmin, retinol, cholesterol

Table 2 Antioxidant concentration and antioxidant activity in circulation at birth

Variable	Well survivors			BPD			Non-survivors		
	n=	Median	Range	n=	Median	Range	n=	Median	Range
Plasma Dmax (µl)	79	80**	13–221	25	92	46–152	39	121	44–258
Caeruloplasmin (mg/l)	69	53	14–440	23	57	14–431	37	67	14–407
Packed cell transfusions	80	0	0–1.0	25	0	0–2.0			
		Mean	SEM		Mean	SEM		Mean	SEM
Vitamin C (µmol/l)	79	80.1*	4.07	25	85.4	7.99	39	105.3	9.66
Retinol (µmol/l)	76	0.53*	0.032	25	0.62	0.075	39	0.70	0.062
α tocopherol (µmol:mol cholesterol)	74	6.85	0.65	25	6.27	0.61	38	7.15	0.81
SOD activity (IU/g Hb)	41	4038	327	19	3625	308	9	2860	468

Significantly different from babies who died (Scheffe test) *p<0.05, **p<0.01.

corrected α tocopherol, and red blood cell SOD activity. Vitamin C, on the other hand, declined in all groups after birth. The magnitude and the timing of the postnatal changes were different between the groups. Retinol showed the greatest increase in the well babies, whereas red blood cell SOD activity showed no significant increase in the well babies. Cumulative data for numbers of packed cell transfusions given to surviving babies are also included in this table. Babies who developed BPD received significantly more transfusions of packed cells up to each time point studied, than those who did not develop BPD.

SURVIVAL ANALYSIS BETWEEN NON-SURVIVORS AND SURVIVORS

Only babies for whom there were complete data (108) were included in this analysis. Gestational age (p<0.001), birthweight (p<0.001), and plasma antioxidant activity (p=0.003) were the most important determinants of survival in these infants. Results were also suggestive of an adverse influence of plasma vitamin C (p=0.058) on survival. Low plasma antioxidant activity (high Dmax) remained a significant predictor of death after correcting for gestational age (p=0.0062). Results of the survival analysis (table 4) showed that for each additional week of gestation, the risk of death was reduced by 34.8% (equation 1). For each additional unit of Dmax—that is µl of plasma required to inhibit the auto-oxidation of lipid substrate— the risk of death was increased by 1.4%. This would mean that a baby with a Dmax of 200 µl at birth would have 4.7 times the risk of death compared with a baby with a Dmax of 50 µl at birth.

LOGISTIC REGRESSION ANALYSES BETWEEN WELL SURVIVORS AND SURVIVORS WHO DEVELOPED BPD

At birth

Complete data from 79 babies out of a total of 105 babies were used for this analysis. Gestational age (p<0.001) and birthweight (p=0.002) were the only significant factors in the development of BPD. The results of the logistic regression analysis showed that for each additional week of gestational age the risk of developing BPD is reduced by 45.3%

Table 3 Biochemical variables and packed cell transfusions over first week of life

Variable	Postnatal age (days)								
	Well survivors			BPD			Non-survivors		
	2	3	8	2	3	8	2	3	8
Dmax (µl)									
n = 45				23			8		
Mean	77*	75	79	91	90	81*	91	80	64*
SEM	3.8	4.0	4.3	4.1	4.3	5.7	12.6	12.7	7.7
Caeruloplasmin (mg/l)									
n = 50				23			10		
Mean	172***	192***	223***	204***	189**	172*	191	184	221
SEM	16.0	19.4	16.7	28.9	22.5	19.3	41.2	30.0	33.5
Vitamin C (µmol/l)									
n = 57				25			12		
Mean	46.1***	42.1***	55.8	57.1**	45.1***	25.3***	63.3	46.3*	26.6**
SEM	3.11	2.78	6.04	3.63	3.50	3.29	14.65	5.39	6.62
Retinol (µmol/l)									
n = 57				25			10		
Mean	0.55	0.61*	0.88*	0.66*	0.67	0.73	0.65	0.61	0.70
SEM	0.037	0.045	0.067	0.053	0.066	0.077	0.076	0.067	0.100
α Tocopherol (µmol/l)									
n = 55				25			10		
Mean	6.8	7.8*	10.1***	7.9*	10.3***	13.2***	10.4***	11.0***	13.3***
SEM	0.48	0.47	0.82	0.91	1.19	1.74	1.10	1.26	1.83
RBC SOD (IU/g Hb)									
n = 41				19			9		
Mean	4152	4089	4255	4248	4301*	4890*	4040*	4218*	4834**
SEM	246	302	263	3265	241	385	78	382	326
Packed cell transfusions									
n = 80				25					
Median	0.48	0.54	0.90	1.00[a]	1.44[b]	2.28[b]			
Range	0–2.0	0–2.0	0–4.0	0–2.0	0–3.0	0–5.0			

Significantly different from day of birth *p<0.05, **p<0.01, ***p<0.001.
Significantly more transfusions than well survivors [a]p<0.01, [b]p<0.001.

Table 4 Independent predictor variables found by survival analysis at birth between non-survivors and survivors with or without BPD

	R	*Exponential (β)*
Gestational age (weeks)	−0.3103	0.6520
Dmax (µl plasma)	0.1439	1.0104

(table 5). The association between birthweight and BPD was lost when corrected for gestational age.

Second day of life

Complete data from 78 babies out of a total of 105 survivors were used for this analysis. The number of packed cell transfusions ($p<0.001$), hours of IPPV ($p<0.001$), high plasma vitamin C ($p=0.008$), higher plasma retinol ($p=0.018$), and a higher ratio of α tocopherol to cholesterol ($p=0.027$) on the second day of life were all significant factors in the development of BPD. The number of packed cell transfusions was the first variable to be entered into the equation by forward selection. After correcting for packed cell transfusions, plasma vitamin C remained a significant independent predictor for the development of BPD. For each packed cell transfusion a baby received, it was 1.6 times more likely to develop BPD (table 5). For each additional unit of plasma vitamin C (1.0 µmol/l), the risk for development of BPD was increased 1.04 times. This would mean that a baby with a plasma vitamin C of 120 µmol/l on the second day of life would have a 16.4 times risk of developing BPD compared with a baby who had a plasma vitamin C of 40 µmol/l at this time.

Third day of life

Complete data from 74 babies out of a total of 105 survivors were used for this analysis. The number of packed cell transfusions ($p<0.001$), hours of IPPV ($p<0.001$), and a higher plasma ratio of α tocopherol to cholesterol ($p=0.01$) were all significant factors in the development of BPD. For each packed cell transfusion a baby received, it was 1.6 times more likely to develop BPD (table 5). After correcting for the number of packed cell transfusions a baby received, no other variable was an independent predictor of BPD.

Eighth day of life

Complete data from 50 babies out of a total of 105 survivors were used for this analysis. The number of packed cell transfusions ($p=0.003$), hours of IPPV ($p=0.006$), low plasma caeruloplasmin ($p=0.03$) and higher plasma vitamin C ($p=0.04$) were all significant factors in the development of BPD. The results show that for each packed cell transfusion a baby received, it was 1.5 times more likely to develop BPD. After correcting for the number of packed cell transfusions, no other variable emerged as an independent predictor of BPD.

Table 5 Independent predictor variables found by logistic regression analysis from birth over first week of life between well survivors and survivors with BPD

	R	*Exponential (b)*
1st day of life:		
Gestational age	−0.3610	0.5472
2nd day of life:		
Number of packed cell transfusions	0.3990	1.6404
Plasma vitamin C (µmol/l)	0.1386	1.0356
3rd day of life:		
Number of packed cell transfusions	0.4205	1.6336
8th day of life:		
Number of packed cell transfusions	0.3517	1.4937

Discussion

Consistent with other work,[8 9] gestational age at birth was the most important predictor of mortality and morbidity in premature infants. Other factors, however, evidently influenced outcome, independent of gestational age.

There is little information regarding circulating concentrations of antioxidants, or antioxidant activity, in the blood of premature infants at birth, although several studies have described antioxidant concentrations in the cord blood of premature infants. Lindeman and coworkers[10] and Berger *et al*[11] have reported higher vitamin C concentrations in the cord blood of premature babies than in babies born at term. Other studies have shown that the concentration of transferrin and caeruloplasmin is lower in cord blood than in plasma from adults, and that a premature delivery is associated with an even lower plasma transferrin concentration.[12 13] Plasma antioxidant activity at birth was an independent risk factor for mortality, despite an association with gestational age. Other attempts to evaluate plasma antioxidant activity in neonates have relied on a measure of plasma peroxyl radical trapping activity (TRAP), which is somewhat higher in cord plasma than in plasma from adults but does not seem to discriminate between babies born prematurely and those born at term.[10] The measure of plasma antioxidant activity used in this study mainly reflects the ability of plasma to keep iron in a form in which it does not readily promote the generation of free radicals. Premature infants who had the lowest antioxidant activities at birth were born with low plasma caeruloplasmin and high vitamin C concentrations. High plasma vitamin C at birth has already been shown to be associated with an increased risk of early mortality.[14] High concentrations of vitamin C in premature infants at birth may inhibit the ferroxidase activity of plasma caeruloplasmin[15] and thereby interfere with the normal binding of iron by transferrin. This may be an important factor in the mortality of this group.

Of all the variables considered at birth, gestational age was the only significant factor in the development of BPD. Although birthweight contributed to the development of BPD, there was no significant effect after correcting for gestational age. None of the biochemical variables measured at birth, nor the administration of antenatal steroids before delivery, contributed to the likelihood of developing BPD.

There were postnatal changes in all groups in plasma antioxidant activity and concentrations of individual antioxidants, the most striking being a fall in vitamin C and an increase in caeruloplasmin, antioxidant activity, α tocopherol and SOD activity. Van Zoeren-Grobben

and colleagues[16] have also reported postnatal changes in some plasma antioxidants, including a decline in vitamin C and an increased α tocopherol, coinciding with a fall in TRAP values. Other workers have observed a postnatal fall in plasma vitamin C in preterm infants[11 17] and have suggested that this impairs antioxidant defence.

The number of packed cell transfusions an infant received was the most important determinant of the development of BPD after birth, on the second, third, and eighth days of life. Mild to moderately severe anaemia often occurs in patients with BPD. It has been suggested that because the delivery of oxygen to the tissues is reduced, packed cell transfusions may improve oxygenation in patients with BPD and anaemia.[18] Babies in this study received packed cell transfusions if they had a low haematocrit. It has been reported before that frequency of blood transfusions is an independent factor in the development of retinopathy of prematurity.[19] Packed cell transfusions may contribute to tissue damage via the introduction of non-protein bound iron from red blood cells which might leak due to damage incurred by packing or storage. This, coupled with an already low plasma transferrin and caeruloplasmin in the baby, may potentiate tissue damage via iron catalysed generation of free radicals. In support of this hypothesis, cord blood plasma from premature infants is reported to contain potentially catalytic iron.[13] We have recently reported the presence of non-transferrin bound iron (NTBI) measured directly, in 50% of plasma samples collected from premature babies over the first week of life.[20]

It is of course possible that the association between packed cell transfusions and outcome may merely be a reflection of severity of the underlying illness, but other factors which might be similarly indicative (the duration of ventilation, for example) do not have the same highly significant correlation.

High vitamin C was a significant predictor of the development of BPD on the second day of life, even after correcting for the number of packed cell transfusions a baby received. As high concentrations of vitamin C can inhibit the ferroxidase activity of caeruloplasmin,[15] packed cell transfusions and high plasma vitamin C may act synergistically to produce tissue damage. The possible pro-oxidant role of vitamin C in this context is at present controversial, and needs to be evaluated further.

The following variables were all significantly associated with the development of BPD before correction for the number of packed cell transfusions: increased plasma retinol on the second day, increased cholesterol corrected α tocopherol on the second and third days of life, lower concentrations of plasma caeruloplasmin and vitamin C on the eighth day of life, and the hours of IPPV at all time points. This might indicate some association between these factors and the number of packed cell transfusions and outcome. However, what is clear is that the most important predictors of BPD, in addition to gestational age, were the number of packed cell transfusions an infant received and high concentrations of plasma vitamin C on the second day of life.

The strength of the predictive value for mortality of poor antioxidant activity at birth raises doubts as to whether treatment after birth could alter mortality statistics. The number of possibly preventable deaths is now considered to be very small.[21] However, the possibility of successful intervention with transferrin or caeruloplasmin at the time of birth, or during delivery, cannot be ruled out as samples in this study were taken at any time up to two hours from birth.

Until further work is done, babies who require treatment for early anaemia of prematurity could be given whole blood and not packed cells. There would clearly be implications for fluid load, which would have to be taken into account, but there would be a reduced likelihood of overloading the iron binding capacity of the plasma and thereby introducing non-protein bound iron with its potential for inducing tissue damage. An increased iron binding capacity, combined with an increase in plasma ferroxidase activity, would be of particular importance to a system in which NTBI has been detected and both transferrin and caeruloplasmin concentrations are low and vitamin C is high. An alternative strategy for neonatal care would be to reduce the need for blood transfusions. There is current interest in the potential of recombinant human erythropoietin (R-HuEpo) in reducing the need for blood transfusions in premature infants.[22] Evidence from premature rabbits suggesting that Epo can reduce lung oxidant damage is compatible with our suggestion that the link between packed cell transfusions and BPD may be causative.[23]

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