High plasma vitamin C concentrations at birth associated with low antioxidant status and poor outcome in premature infants

K M Silvers, A T Gibson, H J Powers

Abstract
Concentrations of circulating antioxidants may be important in the aetiology of disease in premature infants. Blood samples were taken from 49 premature infants within two hours of birth. Plasma ascorbic acid, caeruloplasmin, and the ability of plasma to prevent lipid peroxidation in vitro were measured.

Plasma ascorbic acid concentrations at birth ranged from 26.3 to 185.4 μmol/l. Plasma antioxidant activity at birth (µ/l plasma required to inhibit lipid peroxidation) showed a strong negative correlation with plasma ascorbic acid and the plasma ascorbic acid to caeruloplasmin ratio. After correcting for gestational age and birth weight, plasma antioxidant activity at birth remained a significant predictor of mortality. Such plasma antioxidant activity is partly determined by the ferroxidase activity of caeruloplasmin. This may be modulated by high concentrations of ascorbic acid which may inhibit the antioxidant activity of plasma and thereby influence outcome.

(Arch Dis Child 1994; 71: F40-F44)

There are a number of disorders which are almost exclusive to those infants who have been born prematurely and who have required supplementary oxygen and mechanical ventilation for survival. The incidence of these disorders, which include chronic lung disease of prematurity, necrotising enterocolitis, and retinopathy of prematurity, is thought to be related to damage by free radicals. The premature infant is particularly susceptible to such damage because of the high concentrations of oxygen required to manage neonatal respiratory disease exacerbated by immaturity of the biochemical systems which exist to minimise free radical damage.

We are particularly interested in those parts of the antioxidant defences in premature infants which are amenable to manipulation – the dietary antioxidants – and a longitudinal study is currently in progress documenting the temporal changes in these factors and the relation between dietary antioxidant status, morbidity, and mortality in high risk premature infants. Before this long term study, a pilot study was conducted during which we observed extremely high plasma concentrations of vitamin C in some infants. We are concerned that these high concentrations may seriously impair antioxidant potential.

Free metal ions can initiate oxidative damage via the generation of free radicals. Ferrous iron is particularly active in this respect and damage by ferrous ions is normally minimised by the iron binding capacity of plasma transferrin and the ferroxidase activity of plasma caeruloplasmin. Concentrations of circulating proteins can be very low in the preterm infant and the damage by ferrous iron may be increased by transfusion of packed cells which will effectively increase concentrations of free iron and reduce transferrin and caeruloplasmin concentrations. This may be further exacerbated by the high concentrations of vitamin C which we have observed as in vitro studies, of lipid peroxidation have shown that the antioxidant activity of caeruloplasmin is impaired at high molar ratios of vitamin C to caeruloplasmin.

The importance of these interactions has not been studied in the preterm infant. We have therefore measured vitamin C, caeruloplasmin, and antioxidant activity of plasma at birth and observed the variation of these with gestational age. We investigated whether plasma antioxidant activity is influenced by the ratio of vitamin C to caeruloplasmin. We also examined the relation between plasma antioxidant activity at birth and mortality.

Methods
SUBJECTS
All infants of less than 36 weeks' gestation requiring intensive care were candidates for this prospective study. Infants were excluded from the study if they had significant congenital malformation or inherited metabolic abnormalities. The study was approved by the combined Sheffield hospital trust ethical committee.

BLOOD SAMPLE COLLECTION AND STORAGE
Whole blood (0.5 ml) was collected from an in situ arterial line into a heparinised tube within two hours of birth. Samples were centrifuged immediately at 800 g for five minutes. Undiluted plasma was stored at −70°C for the measurement of caeruloplasmin and antioxidant activity. Aliquots were stored at the same temperature with
5% metaphosphoric acid (1:9, v:v) for the measurement of vitamin C. During collection, storage, and sample preparation, light exposure was minimised to reduce the likelihood of photodegradation.

MEASUREMENT OF PLASMA ANTIOXIDANT ACTIVITY
The antioxidant activity of plasma was measured in vitro as the ability of plasma to inhibit the peroxidation of lipids. The method used was a modification of that described by Sullivan and Newton using rat brain homogenate as the substrate for lipid peroxidation.

PREPARATION OF STANDARD HOMOGENATE
Rat brains were carefully removed and stored at −70°C. After thawing, the meninges were stripped and all blood washed off in ice cold isotonic saline. The tissue was then homogenised for two minutes in a Waring blender (model B68L29) in ice cold phosphate buffered saline, pH 7.4 (1:5, w:v). The resulting homogenate was centrifuged for 15 minutes at 800 g and the supernatant was stored in 5 ml aliquots at −70°C.

ESTIMATION OF ANTIOXIDANT ACTIVITY
A 40 μl volume of plasma was added to 2 ml ice cold brain homogenate (~1:20, w:v, in phosphate buffered saline, pH 7.4). The mixture of plasma and homogenate was serially diluted with an equal volume of ice cold homogenate six times. Each dilution was incubated and agitated at 37°C for one hour. Controls containing either homogenate alone (control 1) or homogenate with 60 μmol/l butylated hydroxytoluene (control 2) were also incubated. Oxidation of brain lipids in control 2 represented oxidation in the presence of a powerful antioxidant and therefore represented minimum oxidation. An 800 μl volume of the incubate was then added to 500 μl 28% trichloroacetic acid and the precipitated protein removed by centrifugation at >1000 g for five minutes.

Substances reactive with thiobarbituric acid were determined by measuring the absorbance at 532 nm after the addition of 250 μl 1% thiobarbituric acid and heating for 30 minutes at 80°C. The residual auto-oxidation was calculated as follows

\[ \text{OD}_{532} \text{sample} - \text{OD}_{532} \text{control 2} \]

\[ \text{OD}_{532} \text{control 1} - \text{OD}_{532} \text{control 2} \]

Residual auto-oxidation was then plotted against the volume of plasma in each dilution. The x intercept of the resulting straight line derived by simple linear regression was the plasma volume in μl (Dmax) required for maximum inhibition of auto-oxidation. A high Dmax value indicates a low antioxidant activity.

PLASMA VITAMIN C
Vitamin C was measured by a fluorimetric technique using a centrifugal analyser with a fluorescence attachment, according to the method of Vuilleumier et al. The assay relies on the conversion of ascorbic acid in a sample to dehydroascorbic acid by ascorbic acid oxidase and the subsequent reaction between phenylenediamine and dehydroascorbic acid to form a fluorescent product.

Plasma was thawed immediately before use and centrifuged at 12,000 g for five minutes. The concentration of vitamin C in each plasma sample was calculated with reference to a standard curve.

MEASUREMENT OF PLASMA CAERULOPLASMIN
Caeruloplasmin was measured using an immunoturbidimetric kit (The Binding Site Birmingham, product code NK045). The assay was automated for the Cobas autoanalyser.

A series of six standards from 13.75 to 440 mg/l was used to prepare a standard graph using a calibrator (pooled lyophilised human serum (preservatives 0.1% sodium azide, 0.1% e-amino-n-caproic acid, 0.1% thiomerital, and 0.1% benzamidine)) of known concentration diluted in isotonic saline with 0.1% sodium azide. Concentrations of caeruloplasmin in plasma samples were calculated using the standard graph and expressed as mg/l.

This measurement was not made on samples from the first 14 infants recruited to the study, but as its potential significance became apparent the measurement was included in the biochemical analyses.

STATISTICS
The degree of association between any two variables was assessed using linear regression analysis. The independent contribution of Dmax to predict mortality over and above gestational age and birth weight was tested using logistic regression. Differences between groups were assessed using the Mann-Whitney U test or the Wilcoxon signed rank test, as appropriate (Statworks).

Results
Forty nine infants were recruited into this study (26 boys, 23 girls). Their mean (SD) gestational age was 29.6 (3.1) weeks (range 24–36) and birth weight 1380 (574) g (range 565–3430).

Table 1 gives the values for plasma vitamin
caeruloplasmin, and antioxidant activity at birth. The data were not normally distributed and therefore medians and ranges are given. All analyses on biochemical data were carried out on log transformed data.

The value of $D_{\text{max}}$ at birth ranged from 13-8 to 257-7 μl and showed a significant negative correlation with gestational age ($p<0.02$, $r=0.338$; fig 1). Plasma vitamin C concentrations at birth ranged from 26-3 to 185-4 μmol/l. A significant negative correlation with gestational age ($p<0.001$, $r=0.456$) was observed. Plasma caeruloplasmin concentrations at birth were often lower than 13-8 mg/l (the lower limit of the assay); in such instances the value was taken to be 13-8 mg/l. Concentrations ranged from 13-8 to 431-4 mg/l with a median of 53-2 mg/l, indicating significant skewness of the distribution. No significant correlation between gestational age and caeruloplasmin concentration at birth was observed.

There was a strong positive correlation between $D_{\text{max}}$ at birth and the plasma vitamin C concentration ($p<0.01$, $r=0.398$; fig 2), but no clear association between $D_{\text{max}}$ and plasma caeruloplasmin (fig 3).

As we have proposed that high concentrations of vitamin C may reduce the antioxidant potential of caeruloplasmin, we have calculated the vitamin C to caeruloplasmin ratio and examined the correlation between this ratio and antioxidant activity. Where plasma caeruloplasmin values were less than the lower limit of the assay, this had the effect of underestimating the ratio. The vitamin C to caeruloplasmin ratio at birth ranged from 0.15 to 13.5 and there was a significant positive correlation with $D_{\text{max}}$ ($p<0.05$, $r=0.349$; fig 4).

Of the 49 infants recruited to this study 41 survived and eight did not. To examine the possibility of an association between mortality and any of the variables measured we compared the measurements in the 41 survivors (group I) with those in the eight infants who died (group II; table 2). The mean $D_{\text{max}}$ at birth was significantly higher in group II than in group I ($p<0.001$). The mean plasma vitamin C concentration at birth in group I was significantly higher than in group II ($p<0.001$). There was no significant difference between the plasma concentrations of caeruloplasmin at birth. The vitamin C to caeruloplasmin ratio was significantly higher in those infants who died than in those who survived ($p<0.01$). Because these variables show a significant relation with gestational age we investigated the independent contribution of $D_{\text{max}}$ to predict mortality using logistic regression. There was a significant effect of $D_{\text{max}}$ ($p<0.01$) and this remained significant even after correcting for gestational age and birth weight (logit risk of dying = $-5.4+0.029(D_{\text{max}})$, SE 0.010).

**Discussion**

Concentrations of vitamin C in the plasma...
of some premature infants at birth were surprisingly high, particularly compared with the normal adult range. There are few reports of plasma vitamin C concentrations in premature infants and none of these are measurements taken in blood from the infant at birth. Lindeman et al reported a mean value of 157 \( \mu \text{mol/l} \) in venous cord blood of premature infants, but found this to be different from the values in cord blood from term infants. Our results show a clear correlation between the degree of immaturity and plasma vitamin C concentrations.

Vitamin C is water soluble and therefore accumulates in the fetus via active placental transport. In adults, excess vitamin C is excreted by the kidney. It is therefore possible that the accumulation of vitamin C postnatally is a result of reduced glomerular filtration in immature kidneys. Work in our laboratory suggests that this may indeed be a contributory factor. The possibility that synthesis of vitamin C occurs in the fetus cannot be ruled out despite the fact that humans are considered to share with guinea pigs an absolute requirement for dietary vitamin C. It is possible that this does not apply to the premature infant, however, as there is some evidence to suggest that vitamin C can be synthesised in the fetal guinea pig.

Plasma caeruloplasmin was generally low at birth compared with term infants and normal adults. This agrees with the findings of Hilderbrand et al, Scott et al, and Sullivan. Caeruloplasmin is able to oxidise ferrous to ferric iron and this constitutes antioxidant activity as ferrous iron can, through Fenton chemistry, initiate the generation of the potent hydroxyl free radical. Blood transfused to premature infants is a potential source of free iron, particularly as packed cells are usually administered, which may increase the risk via a reduction in plasma antioxidants.

In addition to its role as an antioxidant, in some systems vitamin C can function as a pro-oxidant. The pro-oxidant and antioxidant functions of vitamin C depend on the system under study and the concentration of vitamin C. Gutteridge reported that the inhibition of the antioxidant activity of caeruloplasmin towards lipid peroxidation in vitro was determined by the vitamin C to caeruloplasmin ratio. Work conducted in this laboratory suggests that at concentrations measured in plasma from premature infants, vitamin C can show 80% inhibition of ferrooxidase activity. The method used for measuring plasma antioxidant activity is considered to be largely a reflection of the ability of plasma to remove trace amounts of iron. The strong positive correlation between the vitamin C to caeruloplasmin ratio and D\text{max} agrees with this interpretation of the assay. The fact that vitamin C showed a strong negative correlation with D\text{max} whereas plasma caeruloplasmin showed no significant association, suggests that vitamin C is a much more important determinant of plasma antioxidant activity than caeruloplasmin in premature infants at birth.

Short gestation was associated with high concentrations of plasma vitamin C, vitamin C to caeruloplasmin ratio, and poor plasma antioxidant activity at birth. Infants of very low gestational age, however, do not all die and analysis has shown that having corrected for these variables D\text{max} remains a strong predictor of mortality. It is therefore surprising that the work described here suggests whether high vitamin C concentrations and poor antioxidant activity of plasma at birth play a part in the aetiology of mortality or whether they should simply be viewed as prognostic indicators. We are currently designing an intervention study with a view to examining the hypothesis that reducing the vitamin C to caeruloplasmin ratio as soon after birth as possible would improve outcome in premature infants.

In conclusion, some premature infants are born with low plasma caeruloplasmin and high vitamin C concentrations. High concentrations of vitamin C in some premature infants at birth may modulate the ferrooxidase activity of plasma caeruloplasmin. This effect may be an important factor in the mortality of this group.

The authors are grateful to the parents of the babies taking part in this study and to the staff of the special care baby unit at the Jesup Hospital for Women for their cooperation. The study was given financial support by the Special Trustees of the Former United Sheffield Hospitals.

---


High plasma vitamin C concentrations at birth associated with low antioxidant status and poor outcome in premature infants.

K M Silvers, A T Gibson and H J Powers

Arch Dis Child Fetal Neonatal Ed 1994 71: F40-F44
doi: 10.1136/fn.71.1.F40