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VITAMIN C AT CONCENTRATIONS OBSERVED IN PREMATURE BABIES INHIBITS THE FERROXIDASE ACTIVITY OF CAERULOPLASMIN

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High concentrations of total vitamin C have been measured in the plasma of premature infants. At these concentrations ascorbic acid inhibited the ferroxidase activity of caeruloplasmin measured directly *in vitro*. The degree of inhibition was dependent on the ratio of ascorbic acid: caeruloplasmin. Values for the ratio of vitamin C: caeruloplasmin measured in premature babies would be predicted to inhibit ferroxidase activity by up to at least 80%. Ferroxidase activity measured in the plasma of premature babies increased from birth but was significantly lower than in plasma collected from adults (<0.001). Plasma ferroxidase activity was correlated with plasma caeruloplasmin concentration and, in premature babies only, showed a negative correlation with the ratio of vitamin C to caeruloplasmin. High levels of vitamin C in premature babies may compromise antioxidant mechanisms and exacerbate oxidant damage.

KEY WORDS: Premature babies, caeruloplasmin, vitamin C.

INTRODUCTION

There is evidence that diseases common in premature infants, - bronchopulmonary dysplasia, retrolental fibroplasia, and necrotising enterocolitis - are at least partly due to damage by reactive oxygen species.[1-4] High concentrations of inspired oxygen, combined with low concentrations of circulating antioxidants, may lead to free radical formation and initiate self propagating pathways of tissue damage. Interest in the role of dietary antioxidants in improving outcome in premature babies has focused on the fat soluble vitamins A and E while the water soluble antioxidant vitamins C and B_2 have been neglected.

We have observed that vitamin C concentrations in plasma taken from premature babies within 2 hours of birth have a wide range, and may reach values close to 200 μmol/l in some babies. This is substantially higher than concentrations normally observed in adult plasma.[5] In addition, caeruloplasmin concentrations are extremely low in the plasma of some babies at birth, with values falling below the lower limit of detection of our assay (13.75 mg/l).[6] This is in agreement with data reported elsewhere.[7,8] In vitro studies have shown ascorbic acid to be an

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important extracellular antioxidant[9,10] but it can also, paradoxically, promote the generation of reactive oxygen species.[11] This pro-oxidant action depends on the presence of trace metal ions and is exploited in ascorbic acid/Fe (III) systems for the promotion of lipid peroxidation.[12] Ascorbic acid is readily oxidised by Fe (III), leading to the formation of Fe (II), which is recognized as being a potential source of free radicals.[13] Despite this pro-oxidant potential, it is most likely that vitamin C acts *in vivo* as an antioxidant in a healthy individual.[14] Whether the same applies to a premature infant remains unclear.

We have measured the antioxidant activity of plasma from premature babies as the ability of the plasma to inhibit lipid peroxidation *in vitro*. This measurement largely reflects the ability of the plasma to minimise the availability of non-transferrin-bound iron. Plasma antioxidant activity demonstrated a negative correlation with vitamin C and the vitamin C:caeruloplasmin ratio. Babies who died had lower plasma antioxidant activity at birth.[15] We hypothesised that the high concentrations of vitamin C observed in some babies was sufficient to inhibit the ferroxidase activity of caeruloplasmin.

MATERIALS AND METHODS

Materials

Human caeruloplasmin, ovotransferrin, ferrous ammonium sulphate, ascorbic acid oxidase, and o-phenylenediamine were obtained from Sigma. Ascorbic acid, sodium acetate trihydrate, (both Analar grade) and HPLC grade acetic acid were obtained from BDH. The kit for measuring caeruloplasmin was from The Binding Site, Birmingham.

Subjects

Ethical approval for this study was obtained from the South Sheffield Research Ethics Committee. Informed verbal consent was obtained from parents before babies were enrolled. All infants had been admitted to the Neonatal Intensive Care Unit at The Jessop Hospital for women. Gestational age at birth ranged from 24 to 36 weeks. Samples from 33 babies provided longitudinal data for plasma vitamin C and caeruloplasmin from birth to 14 days postnatally. 15 healthy adult volunteers between 25 and 50 years and a further 17 premature babies provided plasma for the measurement of plasma ferroxidase activity and investigation of the association with the ratio of plasma vitamin C: caeruloplasmin. Blood samples were collected from the babies as part of routine care at different postnatal ages, some babies provided a sample on more than one occasion. Samples were collected into lithium heparin and processed immediately.

Ferroxidase Activity of Caeruloplasmin

The ferroxidase activity of caeruloplasmin was investigated using a method based on that described by Johnson *et al.*[16] and developed by Gutteridge.[12] The method was modified to produce the range of concentrations of ascorbic acid and caeruloplasmin that we have observed in premature infants. In principle the method depends on the ability of caeruloplasmin to oxidise ferrous iron. The ferric iron produced

is bound by ovotransferrin to form a complex which absorbs light at 460 nm. The method was automated for the Cobas BioAutoanalyser and used the following concentrations in the final reaction mixture: 38–300 mg/l caeruloplasmin; 5 mg/ml ovotransferrin; 120 μmol/ideoxygenated ferrous ammonium sulphate; 50–300 μ mol/l ascorbic acid; 0.2 mol/l sodium acetate buffer, pH 6.0. 50 μl caeruloplasmin were incubated at 30°C for 180 seconds with ovotransferrin and ascorbic acid, and the reaction was initiated by 20 μl ferrous ammonium sulphate. The absorbance at 460 nm was monitored every 10 seconds for 80 seconds and the rate of change determined by linear regression of absorbance against time. A reagent blank was included and this was subtracted from each reaction rate. Reactions were performed in replicates of 6. The inhibitory effect of ascorbic acid on ferroxidase activity was calculated by comparison with rates of reaction in the absence of ascorbic acid.

Ferroxidase Activity of Human Plasma

The measurement was performed on fresh plasma diluted in 4 parts (adult) or 2 parts (neonate) water. All reactions were measured at least in triplicate.

Vitamin C

Vitamin C was measured as the sum of ascorbic acid and dehydroascorbic acid in plasma samples stored at −70°C with 9 vols. 5% metaphosphoric acid. A fluorometric method was used which had been automated for the Cobas BioAutoanalyser.[17] The use of the term vitamin C in reference to this measurement implies the sum of ascorbic acid and dehydroascorbic acid.

Caeruloplasmin

Caeruloplasmin was measured in plasma stored at −70°C using an immunoturbidometric assay in kit form (The Binding Site, Birmingham - product code NK045). The assay was automated for the Cobas BioAutoanalyser.

Statistics

Data were stored on Excel and analysed using Statsworks. Analysis of variance was used to evaluate the effect of vitamin C on the ferroxidase activity of caeruloplasmin *in vitro*. Student's t-test was used to compare ferroxidase activities in adult and baby plasma. Regression analyses were used to investigate associations between variables.

RESULTS

Ferroxidase Activity of Caeruloplasmin

The reaction was linear over the measurement period except at the highest concentration of caeruloplasmin, for which the reaction rate was determined over 40 seconds. The association between caeruloplasmin concentration and the rate of the reaction was linear (∂ OD/minute = 0.0147 + 0.179. caeruloplasmin (mg/l); $P < 0.001$). The presence of ascorbic acid at 300 μmol/l had no measureable effect

TABLE 1
Ferroxidase activity † of caeruloplasmin in the presence of different concentrations of ascorbic acid

Ascorbic acid (μmol/l)	Caeruloplasmin (mg/l) 38 Mean (SD)	75 Mean (SD)	150 Mean (SD)	300 Mean (SD)
0	0.015 (0.0008)	0.027 (0.0041)	0.042 (0.0061)	0.062 (0.0034)
50	0.011 (0.0005)	0.020 (0.0037)	0.038 (0.0074)	0.055 (0.0017)
100	0.007 (0.0010)	0.014 (0.0003)	0.037 (0.0043)	0.061 (0.0021)
150	0.004 (0.0009)	0.010 (0.0003)	0.026 (0.0008)	0.060 (0.0012)
200	0.003 (0.0005)	0.008 (0.005)	0.021 (0.0004)	0.052 (0.0009)
250	0.002 (0.0008)	0.006 (0.0007)	0.018 (0.0026)	0.049 (0.0015)
300	0.002 (0.0010)	0.006 (0.0005)	0.016 (0.0019)	0.049 (0.0015)

† Activity is expressed as change in OD_{460nm} per minute. The mean of 6 replicates are given.
A two-way ANOVAR showed a highly significant effect of ascorbic acid and caeruloplasmin on ferroxidase activity, and a significant interaction of these two factors ($P < 0.001$)

on the pH of the incubation medium and neither caeruloplasmin nor ascorbic acid contributed to the absorbance at 460 nm.

Table 1 shows the rate of the ferroxidase reaction, expressed as the rate of change in the absorbance at 460 nm, in the presence of different concentrations of ascorbic acid and caeruloplasmin. Each value is the mean of six measurements. For

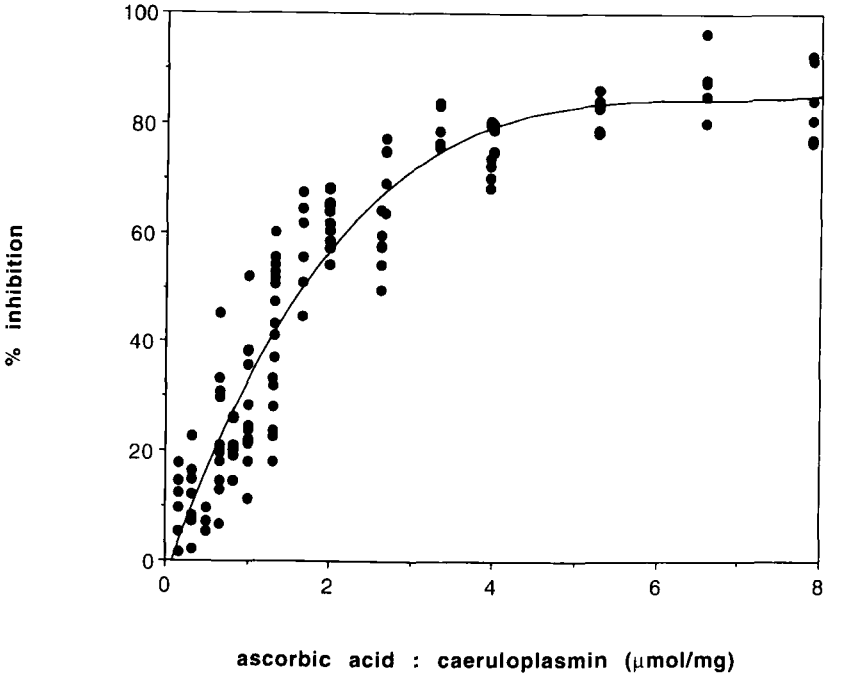

FIGURE 1 The effect of ascorbic acid on ferroxidase activity. Inhibition is calculated with reference to activity without added ascorbic acid.

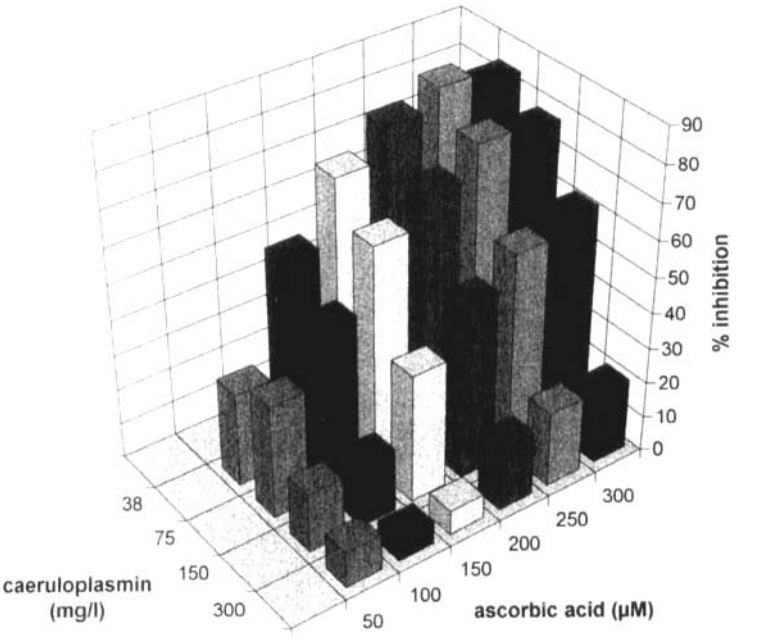

FIGURE 2 The inhibition of ferroxidase activity by ascorbic acid at different concentrations of caeruloplasmin. Each result is the mean of 6 determinations.

each concentration of caeruloplasmin, increasing the concentration of ascorbic acid was associated with a fall in the ferroxidase activity. Two-way analysis of variance revealed a highly significant effect of caeruloplasmin ($P < 0.001$) and ascorbic acid concentration ($P < 0.001$) on ferroxidase activity, with a significant interaction of the two variables ($P < 0.001$).

Figure 1 shows how the inhibitory effect of ascorbic acid on ferroxidase activity is a function of the ratio of ascorbic acid: caeruloplasmin (μmol:mg). The greater this ratio the greater the inhibition of ferroxidase, with 80% inhibition at a ratio of 5 or above. The relationship is best described by a 4th order polynomial, where % inhibition $= -12.149 + 50.642x - 10.772x^2 + 1.0787x^3 - 0.04156x^4$ (x = vitamin C:caeruloplasmin).

Figure 2 shows the relationship between ferroxidase activity and the concentration of ascorbic acid and caeruloplasmin. Inhibition is greatest when caeruloplasmin concentrations are low and ascorbic acid concentrations high.

Figure 3 shows the mean values for the ratio of vitamin C : caeruloplasmin measured in plasma samples collected from 33 babies on the day of birth and at intervals up to postnatal day 14. The values at birth ranged from 0.18 to 13.48 and the mean value (3.08) was significantly higher than at any time thereafter (Student's t-test, $P < 0.001$). 10 babies had a ratio of 4 or above on the day of birth.

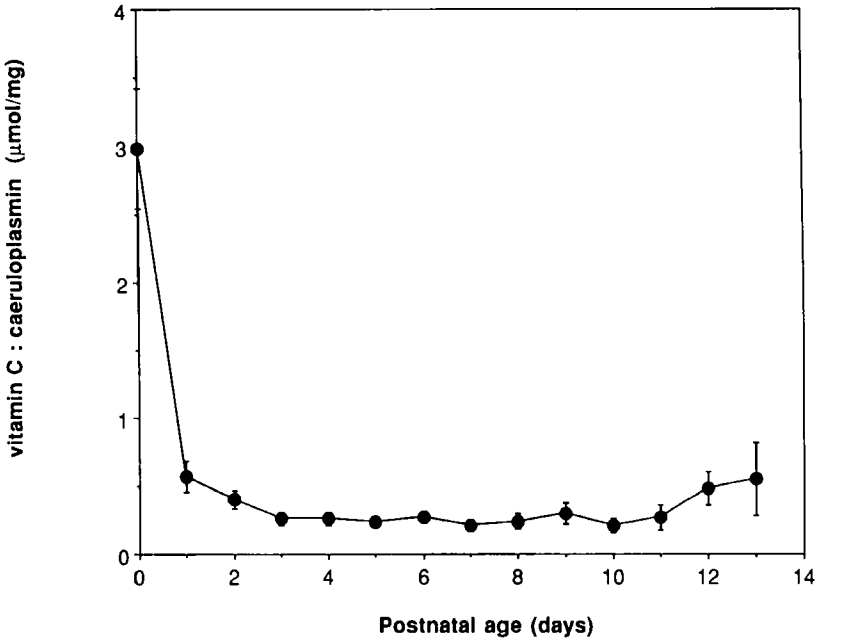

FIGURE 3 The ratio of vitamin C : caeruloplasmin in the plasma of 33 premature babies measured from birth over the first 13 days of life. Mean values ± SEM are given.

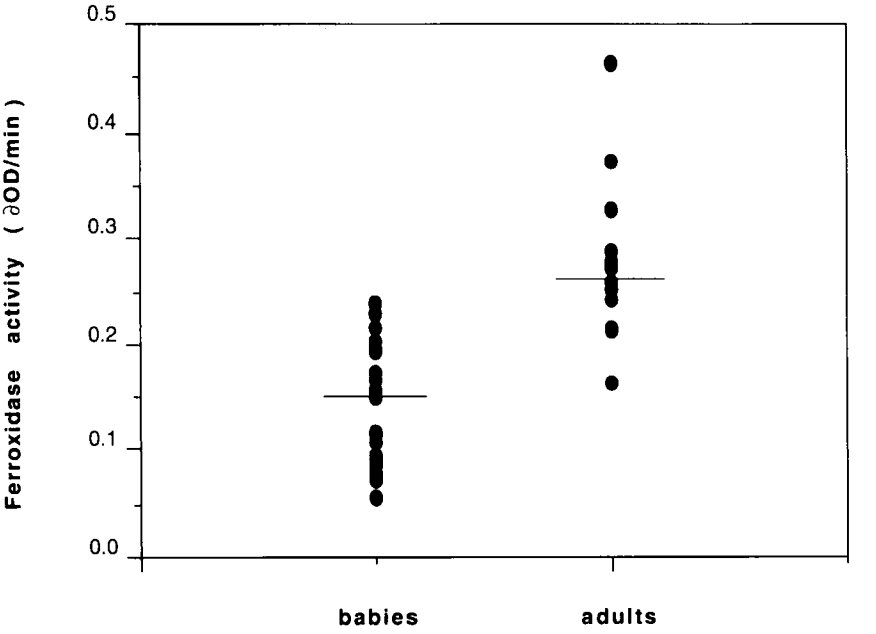

FIGURE 4 Ferroxidase activity in the plasma of premature babies compared with adults. Activity is expressed as the change in OD_{460nm} per minute, and the mean values are shown as a horizontal line.

Ferroxidase Activity of Plasma

Figure 4 shows the ferroxidase activity in fresh plasma collected from 15 healthy adults in comparison with activities measured at various times postnatally in fresh plasma from 17 babies born between 24 and 36 weeks gestation. Blood samples were collected at times between birth and 20 postnatal days. Ferroxidase activity is expressed as the change in optical density (460 nm) per minute. One baby diagnosed with bronchiolitis (RSV + ve) had an elevated caeruloplasmin concentration (218 mg/l) and a correspondingly high ferroxidase activity (0.604 OD units/min.). These values were not included in analyses. Ferroxidase activity in baby plasma ranged from 0.056 to 0.238 with a mean of 0.139. In contrast, values in plasma from adults ranged from 0.164 to 0.466 with a mean of 0.290. These values were significantly different ($P < 0.001$, Student's t test). None of the adult control subjects were pregnant and none were smokers. 4 of 15 were taking oral contraceptives but there were no differences between these subjects and remaining control subjects. There was a positive correlation between plasma ferroxidase activity and caeruloplasmin concentration in adults ($r = 0.785$, $P < 0.001$) and babies ($r = 0.725$, $P < 0.001$), and, in babies only, a negative correlation with the ratio of vitamin C to caeruloplasmin ($r = 0.444$, $P < 0.05$). Ferroxidase activity in plasma of premature babies increased with increasing postnatal age (Figure 5) but did not reach the mean value recorded in adults.

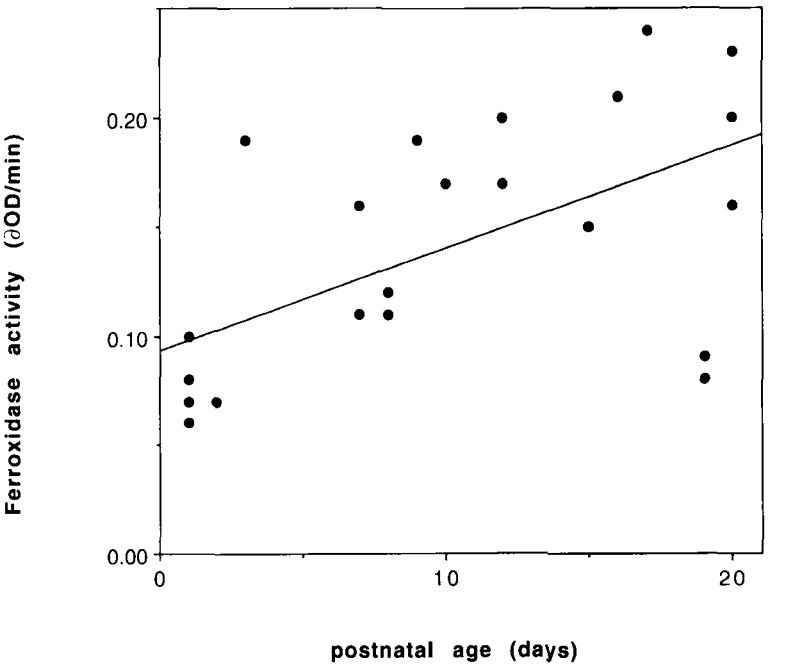

FIGURE 5 Ferroxidase activity in the plasma of premature babies over the first 20 days of life after birth. Regression equation: ferroxidase activity = 9.3037e-2 + 4.6912e-3.(postnatal age) $r = 0.583$ ($P < 0.01$)

DISCUSSION

This *in vitro* experiment clearly demonstrates substantial inhibition of ferroxidase activity of caeruloplasmin in a situation where concentrations of ascorbic acid are high and caeruloplasmin low. Gutteridge[12] demonstrated that lipid peroxidation stimulated by mixtures of iron and ascorbic acid could be inhibited by caeruloplasmin only if the molar ratio of ascorbic acid to caeruloplasmin was low, but an inhibitory effect of ascorbic acid on ferroxidase activity could not be measured directly. He concluded that at physiological concentrations of plasma vitamin C the ferroxidase activity of caeruloplasmin was unlikely to be inhibited.

Some babies born prematurely have high concentrations of vitamin C, and in the neonatal period plasma vitamin C levels can rise even further to achieve values up to 3 times the upper limit seen in adults.[18] Low plasma levels of caeruloplasmin have been observed in premature babies at birth[8] and in cord blood.[7] Very low caeruloplasmin levels, in combination with high plasma vitamin C seen in some premature babies at birth produce ratios of vitamin C:caeruloplasmin as high as 14. The *in vitro* data demonstrated an 80% inhibition of ferroxidase activity at an ascorbic acid: caeruloplasmin ratio of 5. A proportion of the plasma vitamin C will be present as dehydroascorbic acid, possibly up to 20% in premature infants,[19] but the high vitamin C to caeruloplasmin ratios evident in the plasma of some babies at birth would produce ascorbic acid:caeruloplasmin ratios that would be predicted to significantly inhibit the ferroxidase activity of caeruloplasmin.

Ferroxidase activity in the plasma of premature babies increased with increasing postnatal age presumably reflecting the postnatal increase in plasma caeruloplasmin and the transient postnatal fall observed in plasma vitamin C. We have shown that the plasma antioxidant activity of premature babies at birth, measured as the ability of plasma to inhibit lipid peroxidation, shows a strong negative association with the plasma vitamin C: caeruloplasmin ratio. The antioxidant activity was lower in babies who died compared with survivors.[15] The system used to measure plasma antioxidant activity relies on the endogenous generation of reactive oxygen species through the presence of traces of metal ions. Although it is difficult to determine the specific effects of the primary plasma antioxidants in such a system, the assay largely reflects the ability of the plasma to minimise the availability of ferrous iron to promote lipid peroxidation.[20] The inhibitory effect of ascorbic acid on ferroxidase activity is considered to act through interference with the redox activity of copper at its active centre.[21] In this in vitro system there may also be competition between ascorbic acid and copper for the redox state of iron, thus influencing the iron ion stimulation of lipid peroxidation. For ascorbic acid to act as a pro-oxidant *in vivo* the presence of non-transferrin-bound iron is mandatory. Halliwell[14] and Lindeman *et al.*[7] have detected non-transferrin-bound iron in the plasma of premature babies and have implicated iron in the pathogenesis of diseases of prematurity.

Our data suggest that the high level of vitamin C in the plasma of some premature babies at birth, in combination with low caeruloplasmin levels, inhibits the ferroxidase activity of caeruloplasmin and impairs the antioxidant activity of the plasma. This action of vitamin C will be facilitated by the presence of non-transferrin-bound iron in plasma and may be a causative factor in the poor outcome of these babies.

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