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FERROUS IONS DETECTED IN IRON-OVERLOADED CORD BLOOD PLASMA FROM PRETERM AND TERM BABIES: IMPLICATIONS FOR OXIDATIVE STRESS

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Redox active iron chelatable to bleomycin is often present in the plasma of cord blood samples taken from preterm and term babies. The low caeruloplasmin and high ascorbate levels in plasma at birth may allow this iron to exist in the reduced ferrous state. In support of this postulate thirteen cord blood samples showing the presence of low molecular mass iron were able to degrade DNA in the presence of bleomycin and plasma.

KEY WORDS: Oxidative stress, chelatable iron, caeruloplasmin, ascorbic acid, ferrous ions, free radicals, cord blood, neonates.

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INTRODUCTION

Redox active iron in biological systems is a pro-oxidant risk factor for the formation of highly reactive and damaging oxygen species such as hydroxyl, alkoxyl, and peroxyl radicals and possibly, as yet uncharacterised, oxo-iron species (reviewed in Gutteridge and Halliwell).[1] Plasma from a high percentage of preterm and term babies has recently been shown to contain low molecular mass iron that can be chelated to bleomycin.[2,3] In addition to increased levels of pro-oxidant iron, neonatal plasma has disturbed antioxidant profiles: a-tocopherol (reviewed in Bracci),[4,5] β-carotene,[5] caeruloplasmin,[2,6,7] and transferrin[2,6] levels are low, bilirubin levels are raised,[3] and ascorbate is initially raised but rapidly falls[5,9,10] which lead to increased oxidative stress.

Ascorbate makes an important antioxidant contribution to plasma[11] provided that it is not destroyed by transition metal ions.[12,13] Such protection is normally provided by the proteinaceous molecules caeruloplasmin, transferrin and albumin.[14] However it was recently shown that the ferroxidase antioxidant activity of caeruloplasmin is inhibited by a high ratio of ascorbate to protein,[15] and that such inhibitory ratios can occur in cerebrospinal fluids.[16] Low plasma levels of caeruloplasmin in neonates[2,6,7] in combination with high ascorbate levels,[5,9] and iron-overload of transferrin[2,3,8]

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prompted us to look for the presence of ferrous ions in cord blood plasma. When cord blood plasma, showing iron-overload, is added to a DNA-bleomycin complex, the DNA is degraded in the absence of any added iron reductant or inhibitor of the ferroxidase activity of caeruloplasmin, suggestive of the presence of ferrous ions.

MATERIALS AND METHODS

All chemicals and reagents were of the highest purity available from the Sigma Chemical Company Ltd, Poole, Dorset and from Fisons Scientific Equipment, Loughborough, U.K.

Total Bleomycin-chelatable Iron

Redox active iron chelatable to bleomycin was determined by the standard assay procedure.[17,18] To the reaction mixture were added DNA (1 mg/ml), bleomycin (1.5 units/ml), Tris buffer, 1M pH 7.4, magnesium chloride 50 mM and the plasma sample. Ascorbic acid 7.5 mM was added to start the reaction and the mixture was incubated at 37°C for 30 min. Iron chelated from the plasma sample by bleomycin was reduced by added ascorbate and able to form an oxo-iron species which degraded DNA with the release of malondialdehyde. Malondialdehyde was measured spectrophotometrically (A532m) after reacting it with 2-thiobarbituric acid (TBA). Adventitious iron in reagents was removed, or decreased, as previously described.[18] Iron was quantitated by reference to pure iron standards.

Speciation of Ferrous Ions with Bleomycin

The bleomycin assay described above has been modified to speciate ferrous ions.[16] To the reaction mixture were added DNA (1 mg/ml), bleomycin (1.5 units/ml) and sodium phosphate buffer 0.5 M pH 7.0, and the plasma sample. The reaction mixture was incubated at 37°C for 30 min together with a blank containing water instead of bleomycin. TBA-reactivity was developed and measured as described above. The results shown for ferrous ions represent an 'activity' value not a quantitative measure[16].

Iron-binding Antioxidant Activity

The ability of plasma transferrin to compete with bleomycin for iron-binding was measured.[19] The method is essentially as described above for total chelatable iron. The blank value (all reagents minus plasma) is taken as 100% DNA damage, and inhibition (I) or stimulation (S) of this value is expressed as a percentage.

Collection of Blood Samples

Umbilical cord bloods were recovered from the separated placentas within 15 min of the babies birth, and placed in lithium heparin tubes. The blood was carefully mixed and centrifuged at 750g, for 10 min, and the plasma removed and stored at −70C under argon, which preserves ascorbate levels, until the time of analysis. Collection of cord blood samples for neonatal antioxidant studies was approved by the ethical committee of the University Hospital of Leiden. Informed consent was obtained from the parents.

All results shown are the mean ± SEM of three or more separate assays.

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TABLE 1
Bleomycin-chelatable iron levels and clinical details of the babies studied.

Patient code	Sex	Gestational age completed weeks	Weight g	Apgar score[+]	Total bleomycin iron μmol/l	Ferrous ion activity*	Iron-binding antioxidant activity %
1	F	29	1470	2/8/10	1.2	7.6	29 (S)
2	F	30	2050	9/10/10	3.9	13.9	98 (S)
3	M	33	2360	7/8/9	3.9	5.8	99 (S)
4	F	34	2540	9/9/10	3.7	4.7	93 (S)
5	F	34	2500	9/10/10	12.0	3.2	302 (S)
6	M	35	3610	8/9/10/	4.9	17.0	123 (S)
7	F	36	3110	9/10/10	3.3	5.9	84 (S)
8	F	36	1985	9/10/10	3.4	7.8	86 (S)
9	M	37	2750	8/9/10	1.5	6.6	37 (S)
10	F	39	3175	9/10/10	3.8	0.8	97 (S)
11	M	40	3190	10/10/10	1.6	0.4	39 (S)
12	M	40	3530	10/10/10	2.5	10.8	62 (S)
13	F	41	3880	9/10/10	1.5	6.4	23 (S)

[+] The APGAR score indicates the clinical condition of the baby at 1/5/10 minutes after birth and is based on points given for respiration, heart rate, colour, muscle tone and response to stimuli. A score of less than 6 at 5 minutes indicates birth asphyxia.

* Ferrous ion 'activity' is a function of the total bleomycin iron undergoing redox cycling with the total amount of ascorbate present, and is expressed in μmol/l of iron. The higher the ascorbate value the higher the activity will be, provided that caeruloplasmin does not oxidise the iron back to the ferric state. Normal adult plasma does not contain bleomycin-chelatable iron or show ferrous ion activity, and inhibits in the iron-binding antioxidant assay to a value of 61%.

RESULTS

Thirteen umbilical cord bloods from preterm and term babies showing the presence of bleomycin-chelatable iron in the plasma (means 3.6 ± 0.8 μmol/l) were included in this study. Clinical details of each baby are summarized in table 1.

All plasma samples from the cord bloods listed in table 1 were able to bring about DNA degradation in the presence of bleomycin at pH 7.0; no other reagents being added. This assay showed that plasma alone could redox cycle iron bound to bleomycin to the ferrous state, since only ferrous ions bound to bleomycin can degrade DNA with the release of TBA-reactive material. As expected, all the plasma samples showing bleomycin-chelatable iron had no iron-binding antioxidant activities, and stimulated damage to DNA.

DISCUSSION

Neonates are known to have a disturbed pro-oxidant antioxidant balance which leads to severe oxidative stress during the first few weeks of life. We have previously suggested[20] that increased oxidative stress, resulting from the change from a low oxygen pressure in utero to a high oxygen pressure at birth, has been retained during evolution as a useful mechanism for destroying unwanted red blood cells. Foetal red blood cells are larger, contain predominantly haemoglobin F, have a shorter life span, contain more polyunsaturated fatty acids, but have less α-tocopherol, glutathione peroxidase, catalase and possibly superoxide dismutase than adult red blood cells.[21,22]

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Two recent studies[2,3] have shown that plasma from neonates can often have high levels of transferrin iron-saturation with the appearance of low molecular mass chelatable iron. We concluded from our previous studies that the low levels of plasma caeruloplasmin[2,6,7] and the high levels of ascorbic acid[5,9] would result in low molecular mass iron being retained in the ferrous state. Depending upon the ratio of ascorbate to caeruloplasmin, ascorbate has the ability to inhibit the ferroxidase activity (oxidation of ferrous ions to the ferric state) of caeruloplasmin.[15] Based on indirect assays of ferroxidase activity we would predict a 40–50% inhibition of ferroxidase activity by the ascorbate caeruloplasmin ratio of cord blood plasma. However, Powers and colleagues[23] have recently directly measured the effect of ascorbate on ferroxidase activity and concluded that neonatal levels of ascorbate can inhibit ferroxidase activity by up to 80%.

Our findings suggest that low molecular mass iron in neonatal plasma, in the absence of sufficient ferroxidase activity, is maintained in the ferrous state by the high levels of ascorbate. An assumption is made here that iron bound to its native biological ligand behaves in a similar way as iron bound to bleomycin. In support of a pro-oxidant form of iron being present in neonatal blood, Kaur and Halliwell have recently observed in vitro hydroxylation of added phenylalanine to occur.[24] The presence of reactive pro-oxidant forms of iron lends support to the proposal that oxidative stress is a teleological phenomenon of the neonatal period which adds to the haemolysis and physiological jaundice characteristic of the first weeks of life.

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