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Antioxidant Protection against Iron Toxicity: Plasma Changes during Cardiopulmonary Bypass in Neonates, Infants, and Children

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Cardiopulmonary bypass surgery is associated with the release of low molecular mass iron, which increases the saturation of plasma transferrin to over 50% in all adult patients treated. In a significant minority, however plasma transferrin becomes 100% iron saturated and non-transferrin bound iron can be detected in the plasma. An iron-saturated transferrin is also a common physiological finding in normal term and pre-term infants at a time when their plasma antioxidants, which protect against iron toxicity and radical scavenging, are profoundly different from those seen in adults. This study was conducted to assess the extent to which antioxidants, which protect against iron toxicity, are altered in neonates, infants, and children undergoing cardiopulmonary bypass surgery.

Keywords: Cardiopulmonary bypass, antioxidants, iron toxicity, reactive iron species, iron binding, iron oxidation, transferrin, caeruloplasmin

INTRODUCTION

Normal human plasma powerfully protects isolated brain tissue lipids from undergoing peroxidation.[1] Such peroxidation of brain tissue lipids *in vitro* was shown to be dependent on the presence of low molecular mass iron (LMrFe) in the homogenate since several iron chelators inhibited peroxidation.[1] The major antioxidants in plasma that protected were shown to reside in two protein fractions.[2] These fractions represented less than 4% of the total plasma proteins and were identified as transferrin and caeruloplasmin.[2,3]

In healthy adults, the plasma transferrin is approximately one-third loaded with iron and the protein retains a considerable ability to bind iron salts. Transferrin's iron-binding capacity ensures that non transferrin bound, or LMrFe,

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is not present in the plasma of normal healthy adults, and that iron is retained and recycled by the body. Transferrin can bind 2 moles of iron per mole of protein, and when iron is correctly loaded on to its high-affinity binding sites, it is not available as a growth factor for micro-organisms, nor is it available as a pro-oxidant.[4–6]

Caeruloplasmin is an acute phase protein that contains most of the copper present in normal human plasma. The cuproprotein has been shown to have oxidase and peroxidase activities *in vitro* (reviewed in Ref. [7]). However, its biological function(s) remain an enigma. A physiological function as a ferrous ion oxidising enzyme (ferroxidase) has been ascribed to caeruloplasmin,[8] which can facilitate the loading of iron into transferrin, and possibly into ferritin.[9] More recently, the ferroxidase[10] and peroxidase[11] activities of caeruloplasmin have been considered important parts of the body's antioxidant defence. When ferric salts are added to normal plasma, in which the transferrin has been iron saturated, and the caeruloplasmin ferroxidase activity inhibited, they are rapidly converted to the reduced ferrous state by plasma iron-reducing molecules such as ascorbate, urate, and protein thiol groups.[12,13] Ferrous ions, at physiological pH values, will transfer electrons to molecular oxygen to form reactive oxygen species, as well as decompose hydrogen peroxide and organic peroxides to form aggressive oxidants such as the hydroxyl and alkoxyl radical respectively. By catalysing the rapid oxidation of ferrous ions to the less reactive ferric state, caeruloplasmin plays an important role as a plasma antioxidant.[7]

Cardiopulmonary bypass (CPB) surgery in adults is associated with a release of LMrFe, which increases the iron saturation of plasma transferrin to over 50% in all patients treated, whilst in a significant minority it leads to 100% iron saturation of transferrin.[14] A fully iron-saturated transferrin, with non-transferrin bound iron detectable in the plasma, is also a common physiological finding in normal term and preterm neonates,[15,16] at a time when their plasma antioxidants are substantially different from those found in adults.[17]

The purpose of this study therefore was to assess changes in antioxidant protection against iron toxicity, that occur in neonates, infants, and children, a patient group particularly at risk of oxidative stress during CPB surgery.

MATERIALS AND METHODS

5,5-dithio-bis(2-nitrobenzoic acid), apotransferrin (human), caeruloplasmin (human), albumin (human) and brain extract (bovine, type VII) were from the Sigma Chemical Company, Poole, Dorset, UK. All other chemicals were of the highest grades available from Fisher Scientific, Loughborough, Leics, UK.

Sample Collection

The study was approved by the Royal Brompton Clinical Research Ethics Committee, and parents of all patients provided informed consent. Blood samples were collected into lithium heparin tubes and sent to the laboratory for immediate separation. Plasma was stored at −20°C until time of analysis, which was no longer than 7 days after separation.

Patients and Clinical Details

Sixty-five paediatric patients undergoing CPB were grouped as follows: neonates (from birth upto 1 month), infants (>1 month–1 year), children (>1 year–5 years) and children (>5–10 years). Clinical details are summarised in Table I.

Total Plasma Thiols

Thiol groups in the plasma were measured using Ellman's reagent (10 mmol/l 5,5′-dithio-bis(2-nitrobenzoic acid) dissolved in 0.1 mol/l sodium phosphate buffer, pH 7.4. The following reagents

TABLE I Summary of clinical details for each age group of patients undergoing CPB surgery

	Neonates	Infants	Children 1–5 years	Children 5–10 years
N	12	26	21	6
Sex (male)	7	11	10	2
Cross clamp time (min)	92.3 ± 7.7 ($n = 11$)	52.3 ± 4.1 ($n = 25$)	53.2 ± 6.9 ($n = 19$)	31.2 ± 5.4 ($n = 6$)
Bypass time (min)	132.7 ± 11.2 ($n = 11$)	83.7 ± 7.7 ($n = 25$)	74.2 ± 8.8 ($n = 19$)	45.3 ± 7.8 ($n = 6$)
Arrest time (min)	23.2 ± 4.5 ($n = 11$)	35.8 ± 9.1 ($n = 6$)	0	0
Mortality ($n =$)	2	5	0	0
Blood cardiplegia	3	12	8	2
Crystalloid cardioplegia	9	14	13	4

were added to clean tubes: 50 µl plasma, 0.55 ml distilled water, 200 µl sodium phosphate saline buffer, pH 7.4 (0.1 mol/l phosphate in 0.15 mol/l NaCl) and 200 µl Ellman's reagent. Blanks were included for each sample substituting distilled water for plasma. The reaction was complete after 15 min at room temperature (25°C), and the resulting chromogen measured at 412 nm. Total plasma thiols were expressed as micromoles per litre of plasma and calculated as nanomoles per milligram of plasma protein, to correct for haemodilution, using a molar absorption coefficient of 13,600 $l \cdot mol^{-1} \cdot cm^{-1}$ for the thiol complex. Total plasma proteins were measured using the Sigma assay kit (Lowry method), and an albumin standard.

Iron-binding Antioxidant Assay

Phospholipid peroxidation is stimulated by the presence of an iron salt and ascorbic acid. When transferrin has available iron-binding sites it will inhibit peroxidation. Conditions are carefully selected to prevent interference by caeruloplasmin.[3] Bovine brain phospholipids were prepared as a liposomal suspension containing 5 mg/ml in 0.15 M NaCl. The following reagents were placed into clean glass tubes: 0.2 ml phospholipid liposomes (5 mg/ml), 0.2 ml sodium phosphate buffer 0.1 M, pH 7.4 and 10 µl of plasma. The tube contents were mixed and the reaction started by adding 20 µl ascorbic acid 7.5 mM and incubating at 37°C for 20 min. The amount of lipid peroxidation occurring was measured as thiobarbituric acid (TBA) reactivity. HCl (0.5 ml) 25% (v/v) and 0.5 ml of 1% thiobarbituric acid (w/v) in 50 mM NaOH were added to each tube and the contents heated at 100°C for 15 min. When cool, the pink chromogen was extracted into 1.5 ml of butan-1-ol and read at 532 nm against appropriate blanks and controls. The percentage inhibition was calculated relative to the control sample (not containing plasma). A standard of apotransferrin was included as previously described.[3]

Iron-oxidising Antioxidant Assay

Phospholipid peroxidation is stimulated by an iron salt and ascorbic acid under conditions which fully saturate plasma transferrin with iron and remove its iron-binding capacity.[3] Under these conditions the ability of caeruloplasmin to catalyse oxidation of ferrous ions to the less reactive ferric state is measured.[3] The following reagents were added into clean glass tubes: 0.2 ml phospholipid liposomes (5 mg/ml), 0.2 ml sodium phosphate buffer 0.2 M, pH 6.5, 20 µl of freshly made ferric chloride 1 mM, and 10 µl plasma. The tube contents were mixed, and the reaction started by adding 30 µl ascorbic acid 0.125 mM and incubating at 37°C for 20 min. TBA-reactivity,

and percentage inhibition were developed and calculated as described above. A standard of pure caeruloplasmin was included as previously described.[3]

Redox Activity of Plasma

This assay measures the non-specific chain-breaking, or peroxidation-enhancing properties of plasma when it is devoid of its iron-binding and iron-oxidising activities.[3] Under our reaction conditions normal human adult plasma stimulates the peroxidation of liposomes.[3] The following reagents were placed into new clean glass tubes: 0.2 ml phospholipid liposomes (5 mg/ml), 0.2 ml sodium phosphate buffer 0.1 M, pH 7.4, 20 µl, ferric chloride 1.0 mM and 10 µl plasma. The reaction was started by adding 20 µl ascorbic acid 7.5 mM and incubating at 37°C for 20 min. Development of TBA-reactivity, and activity calculations were performed as described above.

RESULTS

Within each patient group, and for each parameter measured, the results have been expressed as a mean ± SEM. Patients showing one or more plasma samples in iron-overload during CPB were compared with those who did not saturate their transferrin at any time point during CPB. None of the children (ages 5–10) undergoing CPB showed plasma iron-overload.

Plasma thiol levels corrected to total protein were often slightly higher in neonates, infants, and children than those seen in normal healthy adults (see Tables II–V). However, they were considerably higher (×2) than values seen in adult

TABLE II Changes in plasma antioxidant activities of neonates ($N = 12$) (up to 1 month) undergoing CPB

	Total thiols nmol/mg protein	Iron-binding antioxidant activity % Inhibition % Stimulation (*S*)*	Iron-oxidising antioxidant activity % Inhibition	Redox activity % Stimulation or % Inhibition (*I*)
Pre-CPB				
LMrFe −ve	5.41 ± 0.39	42.3 ± 7.1	60.6 ± 5.5	14.3 ± 3.8
LMrFe +ve	6.62 ± 0.95	19.0 ± 14.4	57.0 ± 8.5	6.3 ± 2.3
P value	0.209	0.209	> 0.999	0.282
CPB on				
LMrFe −ve	5.59 ± 0.65	26.7 ± 5.2	37.2 ± 6.2	9.2 ± 5.9
LMrFe +ve	5.66 ± 0.89	16.3 ± 13.0	33.0 ± 14.8	1.0 (*I*) ± 6.7
P value	0.864	0.600	0.727	0.282
Cross clamp off				
LMrFe −ve	5.92 ± 0.57	27.6 ± 6.9	50.0 ± 5.8	2.25 ± 4.3
LMrFe +ve	7.31 ± 1.49	4.0 ± 7.0	42.0 ± 24.0	1.00 ± 6.0
P value	0.436	0.056	0.711	> 0.999
CPB off				
LMrFe −ve	5.57 ± 0.4	24.3 ± 5.5	46.1 ± 5.3	10.7 ± 3.7
LMrFe +ve	7.32 ± 1.0	1.7% (*S*) ± 6.36	45.0 ± 9.1	6.7 ± 2.0
P value	0.209	0.024	0.864	0.282
2 h post-CPB				
LMrFe −ve	6.42 ± 0.42	27.1 ± 5.2	58.9 ± 4.1	12.1 ± 2.8
LMrFe +ve	7.63 ± 1.32	7.5 ± 9.5	62.0 ± 8.0	5.0 ± 8.0
P value	0.582	0.178	0.909	0.327

P values show comparisons between samples which showed non-transferrin bound iron (LMrFe +ve) and those which do not have non-transferrin bound iron present (LMrFe −ve).

*Plasma samples stimulate lipid peroxidation when the transferrin is fully saturated and LMrFe is present. The results presented here, however, show a mean value for patients showing one or more plasma sample in iron overload at any given timepoint during bypass. For example pre-CPB only one neonate sample showed LMrFe.

TABLE III Changes in plasma antioxidant activities of infants ($N=26$) (1 month–1 year) undergoing CPB

	Total thiols nmol/mg protein	Iron-binding antioxidant activity % Inhibition % Stimulation (S)	Iron-oxidising antioxidant activity % Inhibition	Redox activity % Stimulation
Pre-CPB				
LMrFe −ve	6.62 ± 0.31	65.4 ± 6.6	70.8 + 3.0	12.0 ± 2.0
LMrFe +ve	6.70 ± 0.66	79.2 ± 7.6	75.4 ± 7.8	10.4 ± 2.3
P value	0.524	0.447	0.650	0.833
CPB on				
LMrFe −ve	6.94 ± 0.4	20.1 ± 2.7	43.9 ± 3.8	8.7 ± 2.3
LMrFe +ve	8.67 ± 2.2	22.0 ± 15.6	43.0 ± 8.9	16.2 ± 1.9
P value	0.488	0.534	0.974	0.060
Cross clamp off				
LMrFe −ve	6.64 ± 0.41	28.8 ± 5.1	54.3 ± 4.0	6.8 ± 2.8
LMrFe +ve	8.43 ± 0.69	11.5 ± 26.5	47.0 ± 8.0	4.5 ± 6.5
P value	0.105	0.549	0.573	0.842
CPB off				
LMrFe −ve	7.58 ± 0.35	33.9 ± 6.4	58.1 ± 3.4	7.2 ± 2.4
LMrFe +ve	7.81 ± 0.23	8.2(S) ± 4.2	47.4 ± 5.1	11.2 ± 1.9
P value	0.530	0.0001	0.097	0.336
2 h post-CPB				
LMrFe −ve	6.98 ± 0.34	39.0 ± 7.0	67.8 ± 3.6	8.8 ± 2.7
LMrFe +ve	7.05 ± 0.23	5.0(S) ± 9.5	59.5 ± 4.8	7.8 ± 2.7
P value	0.736	0.0013	0.221	0.597

TABLE IV Changes in plasma antioxidant activities of children ($N=21$) (1–5 years) undergoing CPB

	Total thiols nmol/mg protein	Iron-binding antioxidant activity % Inhibition	Iron-oxidising antioxidant activity % Inhibition	Redox activity % Stimulation
Pre-CPB				
LMrFe −ve	6.75 ± 0.29	73.7 ± 5.4	81.7 ± 2.0	7.4 ± 1.8
LMrFe + ve	5.08 ± 1.05	90.3 ± 9.7	87.3 ± 5.0	16.7 ± 7.5
P value	0.125	0.153	0.356	0.185
CPB on				
LMrFe −ve	6.84 ± 0.46	29.8 ± 4.9	55.8 ± 2.2	8.2 ± 3.0
LMrFe +ve	6.84 ± 1.77	35.0 ± 14.2	60.7 ± 4.2	13.7 ± 4.2
P value	> 0.999	0.814	0.221	0.262
Cross clamp off				
LMrFe −ve	7.84 ± 0.34	31.5 ± 5.2	58.4 ± 2.3	3.3 ± 2.2
LMrFe +ve	8.18 ± 1.39	12.0 ± 9.7	66.7 ± 1.9	10.3 ± 1.2
P value	0.634	0.138	0.109	0.211
CPB off				
LMrFe −ve	7.5 ± 0.4	34.5 ± 5.5	65.4 ± 2.3	6.7 ± 2.8
LMrFe +ve	7.8 ± 1.14	27.7 ± 12.6	72.0 ± 4.6	17.0 ± 6.1
P value	0.765	1.0	0.258	0.216
2 h post-CPB				
LMrFe −ve	6.77 ± 0.31	27.4 ± 6.8	72.0 ± 3.3	6.4 ± 2.3
LMrFe +ve	7.03 ± 0.87	12.01 ± 12.3	80.0 ± 3.6	10.7 ± 4.1
P value	0.937	0.287	0.371	0.469

TABLE V Changes in plasma antioxidant activities of children ($N=6$) (5–10 years) undergoing CPB

	Total thiols nmol/mg/protein	Iron-binding antioxidant activity % Inhibition	Iron-oxidising antioxidant activity % Inhibition	Redox activity % Stimulation
Pre-CPB	6.75 ± 0.74	61.0 ± 10.6	78.5 ± 3.0	12.8 ± 4.2
CPB on	6.49 ± 0.51	25.2 ± 4.3	64.3 ± 4.0	8.5 ± 4.7
Cross clamp off	7.04 ± 0.89	29.5 ± 9.9	70.0 ± 4.1	10.3 ± 7.4
CPB off	6.9 ± 0.72	21.0 ± 4.4	63.5 ± 5.1	9.7 ± 4.4
2 h post-CPB	7.89 ± 0.58	28.8 ± 8.2	78.2 ± 1.7	15.6 ± 3.8

patients undergoing CPB (3.80 ± 0.22 nmol/mg protein).[18] As previously observed[18] total thiol values corrected to total protein tended to increase during CPB so that all values in neonates, infants and children were higher at the end of bypass than before surgery (Tables II–V).

Iron-binding antioxidant activities are a reflection of the plasma transferrin iron-binding capacity, which is dependent on its percentage saturation with iron. Patients showing an iron-saturated transferrin, with the presence of LMrFe in their plasma (at any time point during CPB), have been grouped together for each time point studied. Before CPB, neonates had substantially lower plasma iron-binding antioxidant activities than those seen in infants and children (Tables III–V) and in healthy adults (86.4% ± 1.14%),[19] or adults undergoing CPB (81.64% ± 4.6%).[19] One of the neonates was in plasma iron-overload before surgery took place, and this decreased the antioxidant activity of the LMrFe positive group by over 50% (Table II).

Iron-binding antioxidant activities decreased substantially in all patient groups partly due to the effects of haemodilution, but also because of the increasing iron saturation of transferrin (Tables II–V). Neonates lost most antioxidant activity, and like the infant group, several plasma samples showed the presence of non-transferrin bound iron and stimulated phospholipid peroxidation (Tables II and III).

The iron-oxidising antioxidant activity of plasma is mainly a function of caeruloplasmin, a protein that is significantly decreased in neonates. Consequently, as expected neonates had the lowest iron-oxidising antioxidant protection (Table II). In all patient groups iron-oxidising activity fell due to haemodilution, but recovered in most cases to pre-surgery values by the end of bypass (Tables II–V).

The 'redox activity' of plasma towards phospholipid peroxidation is a reflection of the balance between molecules that stimulate iron-driven peroxidation and those that inhibit it. During CPB neonatal plasma (from the LMrFe positive patients) became inhibitory on one occasion (Table II), although this did not appear to reflect changes in plasma thiol content (Table II).

DISCUSSION

Iron is a transition metal catalyst essential for life in oxygen. It is used to transport electrons and oxygen and plays a key role at the active centre of oxidases, oxygenases and antioxidants. To avoid unwanted electron transfers, iron is normally tightly sequestered and safely transported around the body by specific iron-binding proteins such as transferrin and lactoferrin, and stored in proteins such as ferritin and haemosiderin. These proteins decrease, or abolish, the ability of iron to participate in redox cycling reactions and hence the generation of reactive forms of oxygen.[20] When iron becomes free of such restraints it is usually present as non transferrin bound iron in a low molecular mass form (LMrFe) which we collectively refer to as 'reactive iron species' (RIS).

Reactive iron species in plasma are considered a pro-oxidant risk factor for the generation of highly reactive and damaging species such as hydroxyl, alkoxyl, and peroxyl radicals, and possibly less well characterised oxo-iron species (reviewed in Ref. [20]).

Plasma from a high percentage of normal term and pre-term neonates has recently been shown to contain LMrFe consistent with the transferrin being fully iron-loaded.[15,16] In addition, neonatal plasma has profoundly disturbed antioxidant profiles showing low levels of α-tocopherol, β-carotene, caeruloplasmin and transferrin, and raised levels of bilirubin and ascorbate although the latter rapidly falls after birth (reviewed in Ref. [17]). We recently observed that CPB surgery in adults resulted in profound changes to plasma iron values[14] leading to iron-overload in many patients.[14,18] At the same time as this occurred, plasma antioxidants that protect against iron toxicity were significantly decreased.[19] A change in the normal balance between pro-oxidants and antioxidants leads to a situation of oxidative stress[21] and the potential for serious tissue damage.

When neonates undergo CPB surgery they experience problems of oxidative stress similar to those seen in the adult population. However, oxidative stress resulting from CPB is superimposed on their physiologically disturbed iron balance and antioxidant protection. Neonates, compared to infants, children and adults are therefore more likely to go into iron-overload during CPB, as well as having the lowest protection against iron toxicity.

Neonates who are not in iron-overload may, therefore, benefit from antioxidant therapy before or during surgery. Since all neonates show disturbances in iron homeostasis during bypass surgery it may be beneficial to add iron-binding proteins such as apotransferrin or apolactoferrin to cardioplegia solutions. This would allow neonates in iron-overload also to receive antioxidants. Since the blood–brain barrier of the youngest neonates may not be fully closed, iron chelators such as desferrioxamine could, in this case, be hazardous.

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