Free Rad. Res., Vol. 21, No. 2, pp. 53–58
Reprints available directly from the publisher
Photocopying permitted by license only

© 1994 Harwood Academic Publishers GmbH
Printed in the United States of America

TRANSIENT IRON-OVERLOAD WITH BLEOMYCIN-DETECTABLE IRON PRESENT DURING CARDIOPULMONARY BYPASS SURGERY

JOHN R. PEPPER, SHARON MUMBY and
*JOHN M.C. GUTTERIDGE

Departments of Cardiothoracic Surgery and Anaesthesia & Intensive Care, Unit of Critical Care, Royal Brompton National Heart & Lung Hospital, Sydney Street, London, SW3 6NP

(Received January 17th, 1994; in revised form February 17th, 1994)

Extracorporeal circulation of blood during cardiopulmonary bypass surgery exposes cells to non-physiological surfaces and shear stress which can activate several regulatory cascades, and neutrophils to release superoxide and hydrogen peroxide. Shear stresses generated by pumps and suction systems cause lysis of red blood cells and the release of haemoglobin. Together the release of reactive forms of oxygen and haemoglobin can lead to the appearance of low molecular mass chelatable iron (bleomycin-detectable iron). All patients undergoing open heart surgery appear to release iron to plasma transferrin, increasing its iron saturation. In 13% of patients, however, the transferrin became fully iron-saturated, and by the end of open-heart surgery we could detect bleomycin-chelatable iron in the plasma. Saturation of transferrin with iron eliminates its iron-binding antioxidant properties, which can result in a stimulation of iron-dependent radical damage to selected detector molecules.

KEY WORDS: Bleomycin-detectable iron, iron-overload, bypass surgery, transferrin saturation, free radicals, reoxygenation injury.

INTRODUCTION

Reactive forms of oxygen such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are transiently formed when ischaemic heart tissue is reoxygenated.[1] The origins of these inorganic molecules remains unclear. However, much evidence suggest they are formed multifactorially through biochemical changes triggered during the period of ischaemia. Superoxide and hydrogen peroxide are poorly reactive molecules known to cause little direct tissue damage in humans. They can, however, be converted to the highly reactive and damaging hydroxyl radical, and possibly to oxo-iron species with similar reactivities, by interaction with redox active iron complexes.[2] The bleomycin assay for chelatable redox active iron was introduced several years ago as a first attempt to detect and measure iron able to participate in free radical reactions.[3,4]

During cardiopulmonary bypass surgery blood is removed from the systemic venous circulation and pumped through a extracorporeal oxygenator before returning to the systemic arterial circulation. Extracorporeal circulation exposes blood to

* Address for correspondence: Professor John M.C. Gutteridge, Oxygen Chemistry Laboratory, Unit of Critical Care, Department of Anaesthesia & Intensive Care, Royal Brompton National Heart & Lung Hospital, Sydney Street, London SW3 6NP, UK.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/13/11
For personal use only.

RIGHTSLINK

severe shear stresses causing the lysis of red blood cells and the activation of several regulatory cascades. Significant 'activation' of blood neutrophils also occurs during extracorporeal circulation[5] causing the release of reactive forms of oxygen such as superoxide and hydrogen peroxide. In the presence of hydrogen peroxide, haemoglobin has the potential to release chelatable forms of redox active iron able to participate in damaging free radical reactions.[6]

In the present study we have examined 23 patients undergoing routine valvular surgery for changes in their plasma iron chemistry. In 13% of patients excessive release of iron, during extracorporeal oxygenation, saturated the plasma transferrin and led to a loss of iron-binding antioxidant activities and eventually stimulation of radical damage to selected detector molecules. This transient iron-overload must be considered an additional, and serious, prooxidant stress during reoxygenation of heart tissue.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/13/11
For personal use only.

MATERIALS AND METHODS

Materials

Bleomycin sulphate and calf thymus DNA, were from the Sigma Chemical Co. Ltd (Poole, Dorset, UK). Radial immunodiffusion plates for transferrin assay were purchased from Behring Hoechst, (Hounslow, UK). Bovine brain phospholipids were prepared as previously described[7] and contained 5 mg phospholipid per ml as a liposomal preparation in 0.15 M NaCl. All other chemicals were of the highest grades available from Fisons Scientific Equipment (Loughborough, UK).

Clinical Blood Samples

Blood samples were taken from a cannula in the coronary sinus from 23 patients (15 male, age range 25–80 years) undergoing aortic valve replacement, by a single operator with or without coronary artery grafting, were analysed at the following time points: (1) Chest open, pre bypass (bypass control), (2) bypass on, (3) cross clamp off, immediately post end of ischaemia, (4) bypass off. The bypass circuit was primed with 1.5 litres of Hartman's solution and the patient cooled to 25°C. Hartman's solution contained 0.19 μmol/l of bleomycin-chelatable iron. Samples, collected into lithium heparin, were stored at 4°C and transported to the laboratory for immediate separation and analysis. Ethical Committee approval was obtained for this study.

Bleomycin-detectable Iron

Iron chelatable to bleomycin was determined as previously described.[3,4] Briefly, the reaction mixture contained DNA, bleomycin and the plasma sample buffered to pH 7.4 with a Tris salt. In the presence of added ascorbate, iron chelated from the plasma sample by bleomycin, degraded DNA with the release of malondialdehyde from its deoxyribose moiety.

Iron responsible for DNA degradation was quantitated with reference to standards by its reaction with thiobarbituric acid (TBA).

RIGHTSLINK

Iron-binding Antioxidant Assays

Plasma transferrin in normal healthy subjects is only one third loaded with iron and retains a considerable iron-binding capacity. This iron-binding potential gives it an important antioxidant activity to inhibit iron-driven free radical reactions.[8] Two assays were used to measure the iron-binding antioxidant activity in plasma[9] employing an organic oxygen radical and an oxo-iron damaging system.

(1) Inhibition of phospholipid peroxidation (organic oxygen radicals). 0.2 ml phospholipid liposomes, 0.2 ml sodium phosphate buffer 0.1 M, pH 7.4 and 10 μl of plasma were mixed in new clean glass tubes and the reaction started by the addition of 20 μl ascorbic acid 7.5 mM. Volumes for blanks, standards and controls were adjusted to 0.5 ml with double-distilled water. The tube contents were incubated at 37°C for 20 minutes in a shaking waterbath. The ability of plasma to inhibit phospholipid peroxidation was expressed as percentage inhibition.
(2) Inhibition of bleomycin-iron damage to DNA (oxo-iron species). 0.4 ml of DNA (1 mg/ml of Chelex resin-treated double-distilled water), 20 μl of bleomycin sulphate 1.5 units/ml, 20 μl of plasma, 0.1 ml magnesium chloride 50 mM, 0.1 ml of Tris buffer 1.0 M, pH 7.4 and 50 μl of ascorbic acid 7.5 mM were placed in new clean polypropylene test tubes. The reaction mixture was incubated at 37°C for 30 minutes in a shaking waterbath. Adventitious iron in reagents (around 1.5 μM) was used to drive the degradation of DNA in the presence of bleomycin and ascorbate, and the ability of plasma transferrin to bind this iron and inhibit DNA degradation was calculated as percentage inhibition. The between batch percentage coefficient of variation of this assay was 4.3%.

The precision of assay system 1 has been described in detail elsewhere.[9] All assays were performed in duplicate and are shown as mean ∓ SEM.

Total Protein and Transferrin Assays

Total plasma proteins were determined using a Sigma kit assay based on the Lowry technique. Plasma transferrin was quantitated by radial immunodiffusion using a polyclonal antibody to pure standards of human apotransferrin.

Total Plasma Iron and Iron-binding Capacity

Total plasma iron and iron-binding capacity were determined using a Sigma kit assay based on the ferrozine spectrophotometric technique. The percentage saturation of transferrin was derived from the measured total iron-binding capacity. This was found to be in close agreement with values calculated from the amount of transferrin present.

Where appropriate, values are corrected to the plasma protein content to adjust for haemodilution.

RESULTS

Of the twenty three patients undergoing routine aortic valve replacement, 13% saturated their plasma transferrin by the end of bypass and had micromolar levels of bleomycin-detectable iron present (Table 1).

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/13/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/13/11
For personal use only.

TABLE 1
Changes in plasma iron chemistry during cardiopulmonary bypass

Subjects/plasma	Pre-Bypass (Control)	Bypass on	Cross clamp off	Bypass off
Bypass patients showing Bleomycin-detectable iron in plasma (3)				
Bleomycin-detectable iron (BLM)μM	0	0	¥	0.840 ± 0.33
Total proteins mg/ml	73.7 ± 3.8	48.5 ± 1.5	44.0 ± 3.89	42.0 ± 1.7
Non-haem iron nM/mg protein	0.303 ± 0.12	0.335 ± 0.07	0.49 ± 0.05	0.54 ± 0.04
Transferrin as % of total protein	2.47 ± 0.19%	1.95 ± 0.35%	2.45 ± 0.85%	2.07 ± 0.18%
% Saturation of transferrin with iron	45.0 ± 16.62%	71.0 ± 26.0%	84.0 ± 39.0%	100%
Antioxidant protection (%Inhibition) organic radicals	59.7 ± 16.2%I	34.0 ± 22.0%I	14%I*	6.67 ± 4.67%S
Antioxidant protection (%Inhibition) oxo-iron species	18.3 ± 7.54%I	13.5 ± 1.5%I	3%I*	14.3 ± 6.1%S
Bypass patients not showing Bleomycin-detectable iron in plasma (20)				
Total proteins mg/ml	66.7 ± 3.9	51.4 ± 3.7	50.6 ± 2.4	53.4 ± 3.3
Non-haem iron nm/mg protein	0.28 ± 0.02	0.330 ± 0.04	0.298 ± 0.02	0.334 ± 0.03
Transferrin as % of total protein	2.90 ± 0.18%	2.60 ± 0.25%	2.70 ± 0.17%	2.64 ± 0.2%
% Saturation of transferrin with iron	37.0 ± 4.41%	50.0 ± 6.43%	46.2 ± 4.7%	49.1 ± 4.91%
Antioxidant protection (%Inhibition) organic radicals	87.6 ± 3.6%I	68.4 ± 6.6%I	59.9 ± 6.1%I	63.5 ± 6.7%I
Antioxidant protection (%Inhibition) oxo-iron species	55.2 ± 4.8%I	43.5 ± 5.1%I	34.6 ± 4.9%I	32.9 ± 5.1%I
Normal healthy controls not undergoing bypass (10)				
Total proteins mg/ml	88.0 ± 2.4			
Non-haem iron nM/mg protein	0.250 ± 0.03			
Transferrin as % of total protein	3.28 ± 0.07%			
% Saturation of transferrin with iron	26.3 ± 3.0%			
Antioxidant protection (%Inhibition) organic radicals	86.3 ± 1.14%			
Antioxidant protection (%Inhibition) oxo-iron species	61.3 ± 1.53%			

Results are shown as means ± SEM ¥BLM iron in only one sample (0.82 μM).
I = % inhibition, S = % stimulation.
*Mean values derived from both inhibition and stimulation values.

RIGHTSLINK

Patients not showing the presence of bleomycin-detectable iron in their plasma, nevertheless increase their iron loading levels to reach a 50% saturation of transferrin during bypass surgery, compared with a 26% saturation seen in normal healthy control subjects (Table 1). This increase in iron-binding during bypass did not appear to result from contamination of the Hartman's solution. Plasma non-haem iron levels (of which bleomycin-detectable iron is a part) increase in the three patients showing bleomycin chelatable iron during bypass surgery (Table), reaching 0.54 nM/mg of protein by the end of bypass. The falling total protein values seen in all patients reflects haemodilution during bypass surgery.

The ability of plasma transferrin to protect against iron-driven free radical damage to phospholipids and DNA was progressively less evident in the plasma from the three patients showing bleomycin-detectable iron, and eventually stimulation of damage to both detector molecules was seen by the end of bypass (Table). As transferrin becomes more saturated with iron it loses its iron-binding antioxidant activity. This is reflected in the bypass patients not showing bleomycin-chelatable iron in their plasma but, who increased their iron loading of transferrin, and were less well able to protect against phospholipid peroxidation and DNA damage compared with normal healthy control subjects (Table 1).

DISCUSSION

During cardiopulmonary bypass surgery, blood is removed from the systemic venous circulation and pumped through an extracorporeal membrane oxygenator before returning to the systemic arterial circulation. Extracorporeal circulation exposes blood to non-physiological surfaces and shear stresses. Circulation and oxygenation can result in the activation of neutrophils, clotting, fibrinolytic, complement and kallikrein/kinin cascades, and to red blood cell lysis. A combination of these events can cause the release of proteolytic enzymes and the formation of reactive forms of oxygen such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Some or all of these changes may contribute to the recognised adverse effects of bypass surgery such as those occurring during the proposed widespread intravascular inflammatory response.[10]

We show here that 13% of patients undergoing routine aortic valve replacement surgery saturate their plasma transferrin by the end of bypass, and have bleomycin-detectable iron in their plasma. Iron that can be chelated by bleomycin, an anti-tumour antibiotic that binds to DNA, is not properly bound to the iron-binding sites of transferrin and hence has the potential to be prooxidant. When the bleomycin-iron complex is redox cycled with ascorbate reactive oxo-iron species are formed which cleave base propenals from the DNA molecule.[11] These rapidly break down to release malondialdehyde which can be measured spectrophotometrically with thiobarbituric acid. Since the reaction on the DNA molecule is site-directed, few naturally occurring antioxidants present in biological fluids interfere with the reaction. Bleomycin is not a strong enough chelator to remove iron correctly loaded on to transferrin or lactoferrin nor iron at the centre of ferritin, haemosiderin or haem proteins.[4]

Normal human blood does not contain low molecular mass iron chelatable by bleomycin (Table 1), since the transferrin is only one third loaded with iron, and retains a considerable iron-binding capacity. This iron-binding capacity gives transferrin an antioxidant function in preventing free radical reactions driven by mononuclear iron ions[8] detected here as organic oxygen radical damage to phospholipids

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/13/11
For personal use only.

RIGHTSLINK

and oxo-iron damage to DNA.[9] Patients not showing the presence of bleomycin-detectable iron, nevertheless, increased their plasma iron loading of transferrin to reach a 50% saturation during bypass surgery. A recent *in vitro* study has confirmed that blood circulated extracorporeally for up to 120 minutes, under mock bypass conditions, can cause release of bleomycin-detectable iron and 'activate' neutrophils.[5] Haemoglobin released from ruptured red blood cells, and hydrogen peroxide released from 'activated' neutrophils have the potential to interact and cause the release of bleomycin-detectable iron.[6] Low molecular mass redox active iron entering the heart (following removal of the cross clamp) during a period of reoxygenation when oxygen toxicity is known to be critical,[12] could lead to the increased formation of highly aggressive and damaging oxidants. This toxicity is further compounded by the partial, (and in some cases total) loss of iron-binding antioxidant protection.

Our continuing studies, on a larger patient population, are directed towards identifying patients at risk during open heart surgery from transient iron-overload, and towards interventions designed to remove, inactivate or decrease the toxicity of released iron before it can participate in the formation of damaging forms of oxygen.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/13/11
For personal use only.

Acknowledgments

JP and SM thank the Wellcome Trust for its generous financial support. JMCG thanks the British Lung Foundation, the British Oxygen Group and the British Heart Foundation for their generous support.

References

1. J.M. McCord (1985) Oxygen-derived free radicals in post ischemic tissue injury. *New England Journal of Medicine*, **312**, 159–163.
2. J.M.C. Gutteridge and B. Halliwell (1989) Iron toxicity and oxygen radicals. In: C. Hershko, ed. Clinical Haematology. Iron Chelating Therapy. Vol 2. No 2. London: Bailliere Tindall. 195–256.
3. J.M.C. Gutteridge, D. Rowley and B. Halliwell (1981) Superoxide-dependant formation of hydroxyl radicals in the presence of iron salts. Detection of 'free' iron in biological systems by using bleomycin-dependant degradation of DNA. *Biochemical Journal*, **199**, 263–265.
4. J.M.C. Gutteridge and Y. Hou (1986) Iron complexes and their reactivity in the bleomycin assay for radical promoting loosely-bound iron. *Free Radical Research Communications*, **2**, 143–151.
5. N.E. Moat, T.E. Evans, G.J. Quinlan and J.M.C. Gutteridge (1993) Chelatable iron and copper can be released from extracorporeally circulated blood during cardiopulmonary bypass. *Federation of European Biochemical Societies Letters*, **328**, 103–106.
6. J.M.C Gutteridge (1986) Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. *Federation of European Biochemical Societies Letters*, **201**, 291–295.
7. J.M.C. Gutteridge (1977) The measurements of malondialdehyde in peroxidised ox-brain phospholipid liposomes. *Analytical Biochemistry*, **82**, 76–82.
8. J.M.C. Gutteridge, S.K. Paterson, A.W. Segal and B. Halliwell (1981) Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. *Biochemical Journal*, **199**, 259–261.
9. J.M.C. Gutteridge and G.J. Quinlan (1992) Antioxidant protection against organic and inorganic oxygen radicals by normal human plasma: The important primary role for iron-binding and iron-oxidising proteins. *Biochimica et Biophysica Acta*, **159**, 248–254.
10. J.K. Kirklin (1991) Prospects for understanding and eliminating the deleterious effects of cardiopulmonary bypass. *Annals of Thoracic Surgery*, **51**, 529–31.
11. L. Giloni, M. Takeshita, F. Johnson, C. Iden and A.P. Grollman (1981) Bleomycin-induced strand-scission of DNA. Mechanisms of deoxyribose cleavage. *Journal of Biological Chemistry*, **256**, 8608–8615.
12. B. Halliwell (1989) Superoxide, iron, vascular endothelium and reperfusion injury. *Free Radical Research Communications*, **5**, 315–318.

Accepted by Prof. B. Halliwell

RIGHTSLINK