Lipid peroxidation, antioxidant enzymes and glutathione levels in human erythrocytes exposed to colloidal iron hydroxide *in vitro*

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Abstract

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The free form of the iron ion is one of the strongest oxidizing agents in the cellular environment. The effect of iron at different concentrations (0, 1, 5, 10, 50, and 100 μM Fe^{3+}) on the normal human red blood cell (RBC) antioxidant system was evaluated *in vitro* by measuring total (GSH) and oxidized (GSSG) glutathione levels, and superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px) and reductase (GSH-Rd) activities. Membrane lipid peroxidation was assessed by measuring thiobarbituric acid reactive substance (TBARS). The RBC were incubated with colloidal iron hydroxide and phosphate-buffered saline, pH 7.45, at 37°C, for 60 min. For each assay, the results for the control group were: a) GSH = 3.52 ± 0.27 μM/g Hb; b) GSSG = 0.17 ± 0.03 μM/g Hb; c) GSH-Px = 19.60 ± 1.96 IU/g Hb; d) GSH-Rd = 3.13 ± 0.17 IU/g Hb; e) catalase = 394.9 ± 22.8 IU/g Hb; f) SOD = 5981 ± 375 IU/g Hb. The addition of 1 to 100 μM Fe^{3+} had no effect on the parameters analyzed. No change in TBARS levels was detected at any of the iron concentrations studied. Oxidative stress, measured by GSH kinetics over time, occurs when the RBC are incubated with colloidal iron hydroxide at concentrations higher than 10 μM of Fe^{3+}. Overall, these results show that the intact human RBC is prone to oxidative stress when exposed to Fe^{3+} and that the RBC has a potent antioxidant system that can minimize the potential damage caused by acute exposure to a colloidal iron hydroxide *in vitro*.

Introduction

Iron deficiency anemia is the most common cause of chronic anemia throughout the world, mainly in developing countries. In addition to blood loss and iron malabsorption, anemia may be caused by inadequate diet (1), blood withdrawn by diagnostic phlebotomy in hospitalized patients (2), hookworm infestation (3), and chronic inflammatory disease (4). Once the diagnosis of iron deficiency anemia is confirmed and the possible causes are identified and treated, replacement of iron stores is indicated. Most patients respond favorably to oral iron preparations. However, when oral supplementation is not possible or fails, the use of parenteral iron is indicated (5). Side effects, which are usually mild, may occur in 25% of patients receiving parenteral iron treatment (6).

On the other hand, iron ions may cause cellular damage by themselves. Several disease states are due to an imbalance between the activities of an oxidant agent and the antioxidant system within the cell (7). One of the strongest oxidizing agents is the free form of iron, Fe^{3+}. Under normal conditions, however, the availability of Fe^{3+} for catalyzing free radical reaction *in vivo* is extremely limited (8,9). It has been shown that oxidation of hemoglobin and lipid peroxidation of human red blood cell (RBC) membranes occur due to iron overload (10). The RBC are intrinsically prone to oxidative stress because they are exposed to high oxygen tension, and have a characteristic structural composition with polyunsaturated fatty acid in the membrane, besides the presence of hemoglobin-bound iron (11,12). However, membranes and cytoplasmic compartments of RBC have an efficient antioxidant mechanism that maintains their integrity. A detoxifying system consisting of reduced glutathione (GSH), superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px) and vitamin E prevents oxidative damage. In addition, there is also a system consisting of NADPH-dependent methemoglobin reductase, ascorbic acid, and glutathione reductase (GSH-Rd), whose main role is the repairing of damage that follows oxidative stress (13). Colloidal iron hydroxide is used for parenteral treatment of severe iron deficiency anemia and its action upon the RBC is not completely established. Furthermore, we are not aware of a comprehensive evaluation of the consequences of acute iron overload on the RBC antioxidant system and its related enzymes in the literature. Thus, the aim of the present study was to determine the response of erythrocytes exposed to different concentrations of colloidal iron hydroxide.

Material and Methods

The protocol for this study was approved by the Ethics Committee of the Botucatu Medical School of the University of the State of São Paulo (UNESP), and the procedures were conducted according to the Helsinki Declaration, as revised in 1983. All subjects gave their informed consent.

The effects of different concentrations of Fe^{3+} were investigated on the normal RBC *in vitro*. Thiobarbituric acid reactive substance (TBARS), and total GSH and oxidized glutathione (GSSG) levels were measured. Activities of SOD, catalase, GSH-Px and GSH-Rd enzymes were determined. All variables were measured at six different iron concentrations (0, 1, 5, 10, 50, and 100 µM Fe^{3+}) provided as colloidal iron hydroxide (NORIPURUM®, BYK, São Paulo, SP, Brazil).

Blood samples were withdrawn by venipuncture from 22 healthy male blood donors (age = 28 ± 12 years) and centrifuged at 3,000 rpm for 20 min. Plasma and the buffy coat were discarded. The RBC were washed three times in cold isosmotic phosphate-buffered saline (PBS; 0.160 M NaH_2PO_4, 0.121 M $Na_2HPO_4.H_2O$, 0.154 M NaCl, pH 7.45) and incubated at 37°C for 60 min with PBS and colloidal iron hydroxide (v/v/v), obtaining six different final iron concentrations: G0 (300/600/0), G1 (300/599/1), G5 (300/595/5), G10 (300/590/10), G50 (300/550/50), and G100 (300/500/100). After incubation, the RBC were washed with PBS to remove iron excess, and subsequently hemolyzed. The hemolysate 1:20 (50 µl of RBC in 950 µl of ice-cold distilled water) was used for hemoglobin (Hb), total GSH, GSSG, SOD and catalase measurements. Water was replaced with a stabilizing solution (2.7 mM EDTA and 0.7 mM 2 mercaptoethanol) for GSH-Px and GSH-Rd activity measurements. A 1:5 (v/v) dilution of the hemolysate in ice-cold distilled water was used for TBARS measurement.

Total Hb was estimated as cyanmethemoglobin using Drabkin's reagent and absorbance measurements at 546 nm (14). GSH

was determined using a kinetic assay involving the continuous reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) by GSH in the presence of NADPH, forming 2-nitro-5-thiobenzoic acid, which was read at 412 nm (15). GSSG was determined in hemolysate previously incubated with 4 vinyl-pyridine for 60 min at room temperature. GSH and GSSG levels are reported as µM/g Hb. Standard curve was determined daily with freshly prepared standard solution of 33 mM GSSG.

GSH-Rd activity was evaluated by monitoring NADPH oxidation at 340 nm and at 37°C (16). The assay mixture contained 1 M Tris, 5 mM EDTA buffer, pH 8.0, 33 mM GSSG and 2 mM NADPH. The enzyme activity (IU/g Hb) was calculated using a millimolar extinction coefficient of 6.22 for NADPH at 340 nm. GSH-Px activity was determined by monitoring NADPH oxidation at 340 nm and at 37°C in an assay mixture containing 1 M Tris, 5 mM EDTA buffer, pH 8.0, 0.1 M GSH, 10 IU/ml GSH-Rd, 2 mM NADPH and 7 mM tert-butyl hydroperoxide. The assay mixture for catalase activity (IU/g Hb) determination contained 1 M Tris, 5 mM EDTA buffer, pH 7.0 and 200 mM hydrogen peroxide (H_2O_2). Disappearance of H_2O_2 was monitored at 240 nm for 30 s (17). SOD activity was measured according to the method described by Beutler (18). Briefly, the reaction is dependent on the presence of superoxide anions that cause the oxidation of pyrogallol. The inhibition of pyrogallol oxidation by SOD was monitored at 420 nm, and the amount of enzyme producing 50% inhibition was defined as one unit of enzyme activity (IU/g Hb). The assay mixture contained 1 M Tris, 5 mM EDTA buffer, pH 8.0, and 10 mM pyrogallol.

Membrane lipid peroxidation was assayed by determining the interaction of thiobarbituric acid (TBA) with the breakdown product of lipid peroxidation under acid pH conditions. The pink chromophore was determined at 535 nm. The assay mixture contained 0.67% TBA, pH 2.0, and TBARS was expressed as nM/g Hb (19).

GSH (L-γ-glutamyl-L-cysteinylglycine), the most prevalent intracellular thiol, is known to function as an antioxidant. Oxidative stress leads to oxidation of GSH to GSSG and formation of protein-SS-protein. Therefore, we may expect a decrease in GSH level when the cell is under oxidizing environment. In the present investigation, oxidative stress was evaluated as the changes in GSH levels over time in hemolysate from RBC incubated previously with different iron ion concentration.

To determine iron-induced oxidative stress, RBC from 14 blood donors were incubated with 0, 5, 10, and 100 µM of colloidal iron hydroxide for 1 h at 37°C (final incubation conditions as described above). After incubation the RBC were washed three times with PBS to remove the iron excess. The RBC were hemolyzed and the hemoglobin level determined. The GSH level in the hemolysate was measured at 50-s intervals throughout 300 s.

All data are reported as mean ± SEM. One-way analysis of variance was used to compare mean values among the six iron-treated groups. Comparisons of GSH levels, over time, in hemolysates of RBC incubated with different iron concentrations were made by the two-way repeated measures analysis of variance and the *post hoc* Tukey test. Significance was set at 5% (20).

Results and Discussion

Mean ± SEM values for variables estimated at different concentrations of Fe^{3+}, in the whole RBC, are shown in Table 1. No significant differences were observed among the groups treated with concentrations up to 100 µM Fe^{3+} for all the variables studied.

The results show that the addition of up to 100 µM colloidal Fe^{3+} to a suspension of normal human erythrocytes does not alter the glutathione redox system and associated enzymes of these cells. Therefore, it may be

Table 1 - Total glutathione (GSH), oxidized glutathione (GSSG), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd), catalase, superoxide dismutase (SOD) and thiobarbituric acid reactive substance (TBARS) levels in red blood cells incubated with six concentrations of Fe^{3+} (G0 = 0 µM Fe^{3+}; G1 = 1 µM Fe^{3+}; G5 = 5 µM Fe^{3+}; G10 = 10 µM Fe^{3+}; G50 = 50 µM Fe^{3+}; G100 = 100 µM Fe^{3+}) at 37°C for 60 min.

All data are reported as mean ± SEM. Superscripts indicate number of samples. The groups were compared by one-way analysis of variance and the level of significance was set at 5%.

Variables	G0	G1	G5	G10	G50	G100
GSH (µM/g Hb)	3.52 ± 0.27[17]	3.63 ± 0.46[13]	3.43 ± 0.39[15]	3.55 ± 0.25[14]	3.69 ± 0.29[13]	3.38 ± 0.22[13]
GSSG (µM/g Hb)	0.17 ± 0.03[17]	0.21 ± 0.04[13]	0.20 ± 0.03[14]	0.24 ± 0.03[14]	0.22 ± 0.04[13]	0.22 ± 0.05[13]
GSH-Px (IU/g Hb)	19.60 ± 1.96[17]	18.10 ± 1.91[17]	19.50 ± 1.93[17]	18.40 ± 1.89[17]	20.10 ± 1.48[17]	16.50 ± 1.99[17]
GSH-Rd (IU/g Hb)	3.13 ± 0.17[22]	3.27 ± 0.25[18]	3.24 ± 0.26[19]	3.30 ± 0.22[18]	3.41 ± 0.28[18]	3.36 ± 0.34[19]
Catalase (IU/g Hb)	394.9 ± 22.8[22]	393.5 ± 23.8[18]	394.3 ± 22.1[20]	374.1 ± 23.8[19]	393.1 ± 28.4[18]	377.5 ± 24.4[18]
SOD (IU/g Hb)	5981 ± 375[20]	6197 ± 465[18]	6081 ± 483[19]	5515 ± 419[18]	6391 ± 488[18]	6276 ± 550[18]
TBARS (nM/g Hb)	13.40 ± 0.98[14]	14.10 ± 1.71[14]	11.70 ± 1.11[14]	13.50 ± 1.43[14]	14.50 ± 1.49[14]	14.90 ± 1.02[14]

assumed that RBC is well equipped to neutralize the oxidative stress imposed by the colloidal iron. In this way, the RBC behaves differently from other cells exposed to the iron ion. For example, hepatocytes from patients suffering from hemochromatosis (21), isolated heart from rats treated with 6 injections of 25 µg Fe^{3+} (22), rat myocardial cells incubated with 0.36 mM ferric ammonium citrate (23), and disrupted brain tissue (11) are morphologically and physiologically altered by lipid peroxidation, increased lysosomal fragility, and oxidation of GSH secondary to iron overload. We did not observe changes in lipid peroxidation or GSH in our experiment. Nevertheless, we have to point out that our experimental conditions were different from those authors.

Besides being potentially prone to oxidative stress, under some circumstances erythrocytes may spontaneously produce significant amounts of the oxidative agent superoxide ($O_2^{\bullet}$) and hydrogen peroxide (24). These features may facilitate and accelerate red cell membrane lipid peroxidation, as can be seen in situations such as microbleeding, where hemoglobin and hemichrome denaturation and iron release occur (11). Despite these characteristics, the observation of clinical or laboratory erythrocyte dysfunction due to oxidative damage is uncommon. RBC

resistance to oxidative stress may be an evolutive characteristic of these highly specialized cells and may quite well explain why they are resistant to the damage caused by *in vitro* iron overload, as observed in the present study. Bartal et al. (25) found an increased malondialdehyde (MDA) and GSH depletion in red cells exposed to t-butyl hydroperoxide. Other investigators have shown increased MDA and GSH-Px activity (26) and increased superoxide dismutase (27) in red blood cells exposed to hydrogen peroxide. However, it should be pointed out that those researchers used t-butyl hydroperoxide and hydrogen peroxide, which are stronger oxidants than the ion Fe^{3+} used in the present study. It is likely that the difference of oxidizing agent may explain the discrepancies observed among the studies.

A relative predominance of Fe^{3+} or Fe^{2+} may lead to different results in the oxidative process (11,28,29). Fe^{3+} induces a slower peroxidation of membranes than Fe^{2+}. Therefore, incubation of RBC with colloidal Fe^{3+} for 60 min may not cause any membrane damage detectable by methods as employed in this work. The maximum concentration of iron (100 µM) used in the present study was 10-fold higher than the normal blood levels of free iron. Borg and Schaich (30) stress that high concentrations of iron (100-200

µM) inhibit iron-dependent oxidative reactions because the ion contributes to the formation of peroxyl and alkoxyl and terminates the peroxidation process. However, Halliwell (31) points out that a putative large availability of iron in the medium increases the probability of oxidative lesions. In the present study, a wide range of iron concentrations was used. Nevertheless, we did not observe any changes in the glutathione redox system associated with the iron concentration. Since the maintenance of enzyme activity was verified for each enzyme studied, we suggest that the enzymatic stability was the result of the strong antioxidant capability of the RBC toward oxidative stress (32,33).

In the experiment, TBARS levels did not differ significantly among the groups exposed to the different concentrations of iron, and the possibility remains that colloidal iron hydroxide induces no measurable membrane lipid peroxidation.

The 60-min period of incubation with iron adopted in this study is similar to that used by Rice-Evans et al. (28), who found a 30% reduction in GSH levels when compared with the levels obtained at the beginning of the incubation period. These authors added H_2O_2 and ascorbate to the system in their *in vitro* assays. In the present study, to simulate the acute iron overload that occurs in parenterally iron-treated patients, those reagents were not added to the medium because they might have induced oxidative lesions by themselves. Therefore, the composition of the incubation medium used by those authors may explain the differences
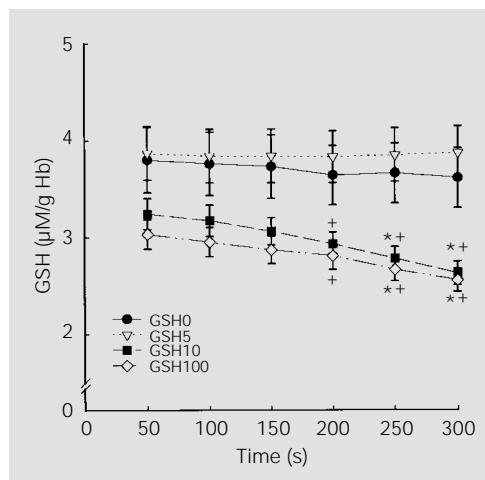

Figure 1 - Time-course changes in total glutathione (GSH) level in a hemolysate of RBC incubated with 0 (GSH0), 5 (GSH5), 10 (GSH10), and 100 (GSH100) µM of colloidal iron hydroxide. Each point represents the mean ± SEM for N = 14. Multiple comparisons were made by two-way repeated measures ANOVA and the post hoc Tukey test. *P<0.05 vs GSH0, +P<0.05 vs GSH5.

between our results and theirs.

Significant decreases in GSH levels over time were observed in the hemolysate from RBC incubated with both 10 µM (GSH10) and 100 µM (GSH100) colloidal iron hydroxide, when compared with RBC incubated with 0 µM (GSH0) and 5 µM (GSH5). No significant difference was observed between the GSH0 and GSH5 groups (Figure 1). The decrement of GSH over time in hemolysate incubated with 10 µM colloidal iron hydroxide suggests that oxidative stress occurs above this concentration. This event does not occur at 0 or 5 µM iron concentration meaning that the oxidative stress is minimal or not present at low iron concentrations.

The present observations show that the normal human RBC has an efficient antioxidant system which is able to reduce the potential damage imposed by acute iron overload in the form of colloidal iron hydroxide.

References

1. Lindebaun J (1966). An approach to the anemia. In: Bennett JC & Plum F (Editors), Cecil Textbook of Medicine. 20th edn. WB Saunders Co., Philadelphia, 823-831.
2. Burns DL, Mascioli EA & Bistrian BR (1996). Effect of iron-supplemented total parenteral nutrition in patients with iron deficiency anemia. Nutrition, 12: 411-415.
3. Stoltzfus RJ, Albonico M, Chwaya HM, Savioli L, Tielsch J, Schulze K & Yip R (1996). Hemoquant determination of hookworm-related blood loss and its role in iron deficiency in African children. American Journal of Tropical Medicine and Hygiene, 55: 399-404.
4. Domachowske JB (1997). The role of nitric oxide in the regulation of cellular iron metabolism. Biochemical and Molecular Medicine, 60: 1-7.
5. Kumpf VJ (1996). Parenteral iron supplementations. Nutrition in Clinical Practice, 11: 139-146.
6. Swain RA, Kaplan B & Montgomery E

(1996). Iron deficiency anemia. When is parenteral therapy warranted? Postgraduate Medicine, 100: 181-193.

7. Halliwell B (1993). The role of oxygen radicals in human disease, with particular reference to the vascular system. Haemostasis, 23: 118-126.

8. Halliwell B & Gutteridge JMC (1988). Iron as a biological pro-oxidant. ISI Atlas of Science: Biochemistry, 1: 48-52.

9. Balla G, Vercellotti GM, Eaton JW & Jacob HS (1990). Iron loading of endothelial cells augments oxidant damage. Journal of Laboratory and Clinical Medicine, 116: 546-554.

10. Rice-Evans C & Baysal E (1987). Iron-mediated oxidative stress in erythrocytes. Biochemical Journal, 244: 191-196.

11. Halliwell B & Gutteridge JMC (1990). Role of free radicals and catalytic metal ions in human disease: an overview. Methods in Enzymology, 186: 1-85.

12. Hatherill JR, Till GO & Ward PA (1991). Mechanisms of oxidant-induced changes in erythrocytes. Agents and Actions, 32: 351-358.

13. Hebbel RP (1986). Erythrocyte antioxidants and membrane vulnerability. Journal of Laboratory and Clinical Medicine, 107: 401-404.

14. International Committee for Standardization in Haematology (1967). Recommendations for haemoglobinometry in human blood. British Journal of Haematology, 13: 71-79.

15. Tietze F (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Analytical Biochemistry, 27: 502-522.

16. Bayoumi RA & Rosalki SB (1976). Evaluation of methods of coenzyme activation of erythrocyte enzymes for detection of deficiency of vitamins B_1, B_2 and B_6. Clinical Chemistry, 22: 327-335.

17. Aebi H (1984). Catalase in vitro. Methods in Enzymology, 105: 121-126.

18. Beutler E (1984). Superoxide dismutase. In: Beutler E (Editor), Red Cell Metabolism. A Manual of Biochemical Methods. Grune & Stratton, Philadelphia, PA, 83-85.

19. Esterbauer H & Cheeseman KH (1990). Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods in Enzymology, 186: 407-421.

20. Snedecor GW & Cochran WG (1989). Analysis of variance: The random effects model. In: Snedecor GW & Cochran WG (Editors), Statistical Methods. Iowa State University Press, Ames, IA, 237-252.

21. Sherlock S (1989). Iron overload states. In: Sherlock S (Editor), Diseases of the Liver and Biliary System. Blackwell, London, UK, 449-459.

22. van der Kraaij AMM, Mostert LJ, van Eijk HG & Koster JF (1988). Iron-load increases the susceptibility of rat hearts to oxygen reperfusion damage. Circulation, 78: 442-449.

23. Link G, Pinson A & Hershko C (1993). Iron loading of culture cardiac myocytes modifies sarcolemmal structure and increases lysosomal fragility. Journal of Laboratory and Clinical Medicine, 121: 127-134.

24. Scott MD, Lubin BH, Zuo L & Kuypers FA (1991). Erythrocyte defense against hydrogen peroxide: preeminent importance of catalase. Journal of Laboratory and Clinical Medicine, 118: 7-16.

25. Bartal M, Mazor D, Dvilansky A & Myerstein N (1993). Iron deficiency: Recovery from in vitro oxidative stress. Acta Haematologica, 90: 94-98.

26. Vives-Corrons JL, Pujades MA & Colomer D (1988). Increase of enzyme activities following the in vitro peroxidation of normal human red blood cells. International Journal of Dermatology, 27: 179-180.

27. Jansson LT, Perkiö MV, Willis WT, Refino CJ & Dallman PR (1985). Red cell superoxide dismutase is increased in iron deficiency anemia. Acta Haematologica, 74: 218-221.

28. Rice-Evans C, Baysal E, Kontoghiorghes GJ, Flynn DM & Hoffbrand AV (1985). Oxidative effects of iron on erythrocytes. Free Radical Research Communications, 1: 55-62.

29. Minotti G & Aust SD (1987). The requirement for iron (III) in the initiation of lipid peroxidation by iron (II) and hydrogen peroxide. Journal of Biological Chemistry, 262: 1098-1104.

30. Borg DC & Schaich KM (1987). Iron and iron-derived radicals. In: Halliwell B (Editor), Oxygen Radicals and Tissue Injury. Proceedings of a Brook Lodge Symposium, August, Michigan, Upjohn/Federation of American Societies for Experimental Biology, 20-26.

31. Halliwell B (1987). Oxygen radicals and metal ions: potential antioxidant intervention strategies. In: Cross CE (Moderator), Oxygen Radicals and Human Disease. Annals of Internal Medicine, 107: 526-545.

32. Frischer H & Ahmad T (1987). Consequences of erythrocytic glutathione reductase deficiency. Journal of Laboratory and Clinical Medicine, 109: 583-588.

33. Gaetani GF, Galiano S, Canepa L, Ferraris AM & Kirkman HN (1989). Catalase and glutathione peroxidase are equally active in detoxification of hydrogen peroxide in human erythrocytes. Blood, 73: 334-339.