

Protective action of deferiprone and deferoxamine in erythrocytes isolated from patients with β -thalassemias

Ação protetora de deferiprona e desferoxamina nos eritrócitos isolados de pacientes com β -talassemias

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*One of the most deleterious consequences of iron overload in thalassemia is the presence of non-transferrin bound iron (NTBI), a free radical that acts as a catalyst for free oxygen radicals, in particular for hydroxyl free radicals (OH \cdot). These radicals oxidize both membrane lipids and proteins causing irreversible damage to biologically important molecules and cellular structures. Treatment with iron chelators has been important to improve survival of these individuals. The aim of this work was the study on the effects of deferoxamine (DFO) and deferiprone (DFP) on erythrocytes under the pro-oxidative action of TBHP isolated from normal individuals and patients with β -thalassemia. The *in vitro* action of deferoxamine and deferiprone on the oxidative metabolism of erythrocytes from β -thalassemic patients treated at the Centro de Hematologia e Hemoterapia do Paraná (HEMEPAR), Brazil, under the pro-oxidative action of TBHP was studied. Methemoglobin concentrations, reduced glutathione (GSH), hemolysis indexes and the enzyme activities of G6-PD and GR were determined. The oxidation indexes were higher in erythrocytes of β -thalassemic individuals than those from normal individuals. Treatment of the normal and β -thalassemic erythrocytes with DFO and/or DFP protected against the formation of GSH promoted by TBHP. Rev. Bras. Hematol. Hemoter.*

Key words: Iron chelators; deferoxamine; deferiprone; erythrocytes.

Introduction

Iron is essential for all organisms, where it participates in some metabolic processes, such as the transport of oxygen by hemoglobin in the erythrocytes, synthesis of DNA and electron transport.¹ However, in higher amounts, it is harmful to the health for the generation of free radicals, which can initiate lipid peroxidations in the membrane, as well as aggression to proteins of tissues and membranes, to enzymes, carbohydrates and DNA.²

Patients with β -thalassemia major have body iron

overload, originated from frequent transfusions and gastrointestinal iron hyperabsorption. Each milliliter of transfused red cells contains approximately 1 mg of iron.¹ There is also an accumulation of additional iron, due to the deficiency of production of normal hemoglobin protein chains.³ The excess of iron is deposited in the endocrine, hepatic and/or cardiac system causing functional damage to the organs.⁴

According to Cohen *et al.*⁵ iron chelator used therapeutically has the objective of lowering the amount of this metal to safer levels, avoiding complications in the

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organism. The ideal properties of the chelators should be specificity and affinity by Fe^{+3} ; efficiency of the chelating action; easy penetration in organs and tissues; no redistribution of the iron; relatively low toxicity; low cost and viability for oral administration. Deferoxamine (DFO) and deferiprone (DFP) has been used in the treatment of β -thalassemic patients. DFP has a lower molecular size than DFO, and penetrates more easily in the cells.⁶

The pro-oxidative action of tert-butyl hydroperoxide (TBHP) has been used to analyze the oxidative stress in isolated erythrocytes.⁷ It can cause metabolic alterations such as unbalance on the homeostasis of intracellular calcium; oxidation of the reduced glutathione, and of proteins thiol groups; damages on DNA molecules; lipid peroxidation; lowering of the intracellular mobility of the membrane proteins.⁸

The understanding of the oxidative stress mechanisms helps to explain many of the processes of cellular lesion and death, especially those related to the hemolytic diseases. The sickle cell anemia, thalassemias and glucose 6-phosphate dehydrogenase (G6-PD) deficiency are among the more frequent genetic anomalies accompanied by oxidative stress. The oxidative action induced by TBHP was observed in erythrocytes from patients with sickle cell anemia by the increase on the content of Heinz bodies, methemoglobin, hemolysis, GSH depletion and lowering activities of the enzymes G6-PD and glutathione reductase (GR).⁷

The aim of this work was the study on the effect of DFO and DFP in isolated erythrocytes from normal individuals and patients with β -thalassemias, under the pro-oxidative action of TBHP.

Materials and methods

Erythrocytes suspensions

Venous blood was collected in CPDA₂ after informed consent, according the Ethical Committee for Research Involving Humans, Setor de Saúde of the Universidade Federal do Paraná. The samples were collected and processed in agreement with the Resolution no. 154 of the Collegiate Directory of the Sanitary Vigilance National Agency (Anvisa) of the Brazil Ministry of Health. It was collected 50 to 100 mL from 6 normal individuals; 10 mL from 5 patients with β -thalassemia minor, and from 10 patients with β -thalassemia major assisted at Centro de Hematologia e Hemoterapia do Paraná (Hemepar), Brazil. From the latter, 3 were regularly treated with DFO, and 7 treated with DFO and DFP. The blood samples were collected before the routine transfusion of the 30-days.

Blood samples with normal and β -thalassemic erythrocytes were centrifuged at 1200 x g for 10 min at 4°C (Hermle centrifuge) and the plasma and the buffy-coat was taken by aspiration. The erythrocytes were then washed three times with 154 mM NaCl in 27 mM phosphate buffer, pH 7.4,

at 1200 x g for 10 min, and resuspended in the same buffer to a 40% cell packed volume.¹¹ Samples of β -thalassemia major erythrocytes were not washed. Aliquots of 1 mL, were centrifuged at 1200 x g for 5 min, and the supernatants were substituted by the same volume of the reagent solutions.

Samples of β -thalassemia major erythrocytes were not washed in order to keep the integrity of DFO and DFP as originally taken from the blood of medicated patients

Erythrocytes incubation with deferoxamine, deferiprone, and tert-butylhydroperoxide (TBHP)

Aliquots of 1 mL of erythrocyte suspensions were incubated with 1-4 mM DFO and/or 3-12 mM DFP in 154 mM NaCl under homogenization, during 60 min (Phoenix HS 22). For the studies on the pro-oxidative effect of TBHP, the erythrocytes suspensions (1 mL) were then incubated with 3 - 5 mM TBHP, and homogenized during 30 min (Phoenix HS 22).

Stock solution of TBHP (Sigma) as 50 mM was prepared in 67 mM phosphate buffer, pH 7.6, with glucose 200 mg/dL.

Determination of Heinz bodies

Heinz bodies were counted according to Beutler *et al.*⁹ modified by Claro *et al.*¹⁰ It was added 25 μL of the treated erythrocytes to 0.5 mL of 67 mM phosphate buffer, pH 7.4 with 200 mg/dl glucose. The mixture was homogenized with the aid of an automatic pipette, and then an aliquot of 25 μL was added to 50 μL of methyl violet (2 mg/dL in 0.73 mg/dL NaCl), on a microscopy slide, under a coverslip. After 5 min, erythrocytes with Heinz bodies were counted using light microscopy (1000 cells per slide). The results are expressed as the percentage of erythrocytes showing more than one violet inclusion, 1-3 μm wide, near the plasma membrane.¹¹

Determination of methemoglobin (MetHb)

MetHb concentration was determined according to Evelyn and Malloy,¹² modified by Beutler *et al.*¹³ It was added 100 μL of erythrocytes suspension in 10 mL of 16 mM phosphate buffer, pH 6.6, and the absorbance was measured at 630 nm against water blank, after 5 min (Shimadzu spectrophotometer). Then, 50 μL of neutralized cyanide (acetic acid 12 g/dL and sodium cyanide 10 g/dL) was added to the cuvette and mixed, and a second reading at 630 nm was made after 5 min. This lysate was then diluted in 66 mM phosphate buffer, pH 6.6 and added of 50 μL potassium ferricyanide (20%) and 50 μL sodium cyanide (10%). The absorbance was measured at 540 nm against the reactant blank, to measure the total amount of hemoglobin in the sample. The methemoglobin concentration was expressed as percentage of total hemoglobin.

Determination of reduced glutathione (GSH)

GSH concentration was analyzed by the method for determination of blood glutathione¹⁴ evaluating the reduction of 5,5'-dithiobis (2-nitro benzoic acid) (DTNB) by sulfhydryl

compounds at 412 nm (Shimadzu spectrophotometer), and expressed in $\mu\text{mole GSH/g}$ of hemoglobin.

Hemolysis

Hemolysis percentage was analyzed by the cyanomethemoglobin method for low hemoglobin concentrations according to Pelissari *et al.*¹⁵ To the hemoglobin-containing supernatant the Drabkins reagent¹⁶ was added, with dilution from 1:3 to 1:110, according to the visual hemolysis degree, from uncolored to brilliant red. This reaction converts hemoglobin to cyanomethemoglobin, and the concentration can be monitored at 540 nm wavelength (Shimadzu spectrophotometer).

Freezing-thawing hemolysates

It was added 0.1 mL of the erythrocyte suspensions to 1.9 mL of EDTA- β -mercaptoethanol,¹⁷ and homogenized, followed by two cycle of freezing-thaw procedure: at -20°C and at room temperature for 5 min each. The hemolysates were kept at 4°C .

Activity of glucose 6-phosphate dehydrogenase (G6-PD)

The G6-PD activity was monitored according to Beutler.¹⁷ It was added 20 μL of the hemolysate (1:20) to 0.98 mL of reaction medium (1 M Tris-HCl, pH 8.0; 5 mM EDTA, 0.1 M MgCl_2 ; 2 mM NADP). It was incubated for 10 min at

37°C and then added 100 μL of 6 mM glucose 6-phosphate. The reaction was monitored at 340 nm (Shimadzu UV 1601) and the enzymatic activity was expressed in IU/g Hb. The molar absorptive coefficient of NADPH is 6.22 L/(mole x cm). One unit is equal to 1 μmole of NADPH formed per min.

Activity of glutathione reductase (GR)

The GR activity was monitored according to Beutler.¹⁷ It was added 10 μL of the hemolysed (1:20) to 0.84 mL of reaction medium (1 M Tris-HCl, 5 mM EDTA, pH 8.0, incubated for 10 min at 37°C , and added 100 μL of 0.033 M, GSSG). It was incubated for more 10 min at 37°C . The reaction was started by addition of 50 μL of 2 mM NADPH, and monitored at 340 nm (Shimadzu UV 1601). The enzymatic activity was expressed in IU/g Hb. One unit is equal to 1 μmole of NADP formed per min.

Statistical analysis

The statistical significance of the experimental data was analyzed using the ANOVA and Tukey tests. A p value < 0.05 was considered to be significant.

Results and discussion

Table I illustrates some biochemical measurements on erythrocytes from normal individuals and from patients with β -thalassemia minor and major.

Table 1. Pro-oxidizer action of TBHP and antioxidant effect of deferoxamine and deferiprone in erythrocytes of normal individuals and of patients with β -thalassemia minor and β -thalassemia major

Erythrocytes	Assays	MetHb* (%)	GSH* (Mmole/g Hb)	Hemolysis* (%)	Heinz bodies* (%)	G6-PD** (IU/g Hb)	GR** (IU/g Hb)	
Normal§ N=6	Control	0	7.5	0.21	0	12.27	7.79	
	TBHP	20	0.15	0.65	9	10.27	7.55	
	DFO	3.2	3.1	0.79	0	7.71	7.94	
	DFP	32.8	3.5	0.82	6.8	7.97	8.12	
	DFO + DFP	21.6	4.62	0.98	-	-	-	
β -Thalassemia minor§ N=5	Control	0	6.79	0.26	5	22.36	9.16	
	TBHP	26.5	0.5	2.74	34	7.74	8.91	
	DFO	19.4	1.34	-	-	-	-	
	DFP	25.9	2.97	-	-	-	-	
	DFO + DFP	31.2	3.74	-	-	-	-	
β -Thalassemia major§§ N=10	A1 N=3	Control	0	8.17	0.57	12	9.76	9.92
		TBHP	26.4	2.85	6.49	43	3.33	9.73
	A2 N=7	Control	0	8.41	0.52	19	10.22	9.44
		TBHP	26.5	3.51	5.6	51	2.71	9.47

Erythrocytes treated with *3 mM TBHP, and ** 5 mM TBHP.

Control - untreated erythrocytes; TBHP -TBHP treated erythrocytes.

§ - Erythrocytes from normal individuals and β -thalassemic minor patients incubated with DFO and/or DFP prior to TBHP treatment.

§§ - Erythrocytes from β -thalassemic major patients under treatment with: A1 - DFO and A2 - DFO + DFP.

- not determined.

It can be observed that under TBHP action there are methemoglobin formation; reduction of the GSH concentration; increase of formation of Heinz bodies and hemolysis percentage.

In erythrocytes from normal individuals the DFO treatment showed a protective effect, where only 3.2% of methemoglobin was observed against 20% of methemoglobin observed with TBHP alone.

β -thalassemic minor erythrocytes were more vulnerable to the pro-oxidative action of TBHP, presenting more than 25% methemoglobin. Samples treated with iron chelators and then submitted to the oxidative action of TBHP, showed methemoglobin concentrations equally high.

Treatments of the normal and β -thalassemic erythrocytes with DFO and DFP protected against the oxidation of GSH promoted by TBHP. According to Breuer *et al.*,¹⁸ DFP removes the iron from the cells, and then it is transferred to the DFO, allowing the regeneration of DFP. Better results were found when DFP was added followed by DFO, prior to the TBHP action. The protective action on GSH was the following: DFO < DFP < DFP + DFO (ANOVA, $p < 0.05$). In the presence of the iron chelators, the GSH seemed to be partially protected from TBHP action. It can be inferred that the DFO or DFO + DFP could assist the organism of β -thalassemic patients to control the pathological mechanisms of oxidative stress, inherent to its condition.

Hemolysis induced by TBHP was much higher in β -thalassemic erythrocytes than in the normal ones. The samples treated with the iron chelators did not present protection against hemolysis.

It was observed 9% Heinz bodies in normal erythrocytes in the presence of TBHP, but its formation was prevented by DFO. However, the DFP did not inhibit the Heinz bodies' formation, a phenomenon consequent to the increase in the methemoglobin concentration. Although generally the individuals with β -thalassemia minor present only discrete anemia or no anemia, some alterations can be evidenced, such as the presence of Heinz bodies, as a result of an exacerbated oxidation. In this work, it was observed formation of about 5% of Heinz bodies in samples of β -thalassemia minor and 10% in samples of β -thalassemia major. Under the pro-oxidative oxidation by TBHP, they presented values of 34% and 43% of Heinz bodies, respectively, for β -thalassemia minor and major. In β -thalassemic major erythrocytes the elevated Heinz body percentages could not be prevented by DFO or DFO/DFP therapy.

The activity of G6-PD was lowered in the concentration of 5 mM TBHP. At that TBHP concentration, samples treated with the DFO and DFP did not recover the level of G6-PD activity. The activities of G6-PD are higher in erythrocytes from β -thalassemic minor. Possible explanations for this fact could be related with metabolic alterations involved in protection on the redox states, such as the GSH levels, and/or with elevated reticulocyte number, because of hemolysis.

In β -thalassemic major erythrocytes, on the other hand, the G6-PD activities were under normality and reduced by the TBHP effect.

Enzyme activities of GR in erythrocytes from β -thalassemic major were higher than the ones not carrying hemolytic illnesses, and did not change either after patients treatment with iron chelators or the addition of TBHP.

The activities of GR and G6-PD are normally higher in β -thalassemics in comparison to the normal individuals, due to necessity of the presence of these enzymes to regenerate the GSH. However, the rates are considered insufficient to regenerate the necessary amount of GSH required for the cells.

The G6-PD regenerates GSH through the generation of NADPH, important for the maintenance of the integrity of certain protein in the mature erythrocytes. The low activities observed for this enzyme could be explained by the increased necessities of the β -thalassemic erythrocytes in keeping the cellular integrity, once that they present trends to the exacerbation of the oxidative processes.¹⁹

More research should be done, in order to better understand the oxidative metabolism of erythrocyte of β -thalassemic patients, especially involving the employment of iron chelators. New contributions are required to offer improvements, not only to guarantee better conditions of life to the β -thalassemic major but also to the β -thalassemic minor whose pathology tends to be minimized by presenting, in general, fewer and lighter signals and detectable symptoms.

Conclusions

β -thalassemia minor and major erythrocytes were more sensible to the pro-oxidative action of TBHP than the normal ones, presenting larger amount of Heinz bodies. Treatments of the normal and β -thalassemic erythrocytes with DFO and/or DFP protected against the oxidation of GSH promoted by TBHP. In erythrocytes from normal individuals but not from β -thalassemics the DFO treatment showed a protective effect against methemoglobin formation promoted by TBHP.

Resumo

Uma das maiores consequências da sobrecarga do ferro na β -talassemia é a presença de ferro não ligado à transferrina (NTBI), um radical livre que age como um catalisador do radical livre do oxigênio, particularmente radical hidroxil (OH \cdot). Estes radicais oxidam os lipídeos e as proteínas da membrana causando danos irreversíveis às moléculas biologicamente importantes e às estruturas celulares. O tratamento com quelantes do ferro é importante para a melhoria da sobrevivência destes indivíduos. O objetivo deste trabalho foi o estudo sobre o efeito da desferoxamina (DFO) e da deferiprona (DFP) em eritrócitos isolados de indivíduos normais e de pacientes com β -talassemias, sob a ação pró-oxidativa de TBHP. Neste trabalho foi estudada a

ação *in vitro* da desferoxamina e o deferiprona no metabolismo oxidativo dos eritrócitos de pacientes β -talassêmicos atendidos no Centro de Hematologia e Hemoterapia do Paraná (Hemepar), Brasil, sob a ação pró-oxidativa de TBHP. Concentrações de metahemoglobina glutatona reduzida, índices de hemólises, atividades das enzimas G6PD e GR foram determinadas. Os índices de oxidação analisados foram maiores nos eritrócitos de indivíduos β -talassêmicos do que nos normais. Tratamentos dos eritrócitos normais e β -talassêmicos com DFO e/ou DFP protegem contra a oxidação de GSH promovida por TBHP. Rev. Bras. Hematol. Hemoter.

Palavras-chave: Quelantes de ferro; desferoxamina; deferiprona; eritrócitos.

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