Impact of iron overload on interleukin-10 levels, biochemical parameters and oxidative stress in patients with sickle cell anemia

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***Objective**: The aim of this study was to evaluate the impact of iron overload on the profile of interleukin-10 levels, biochemical parameters and oxidative stress in sickle cell anemia patients.*

***Methods**: A cross-sectional study was performed of 30 patients with molecular diagnosis of sickle cell anemia. Patients were stratified into two groups, according to the presence of iron overload: Iron overload (n = 15) and Non-iron overload (n = 15). Biochemical analyses were performed utilizing the Wiener CM 200 automatic analyzer. The interleukin-10 level was measured by capture ELISA using the BD OptEIAT commercial kit. Oxidative stress parameters were determined by spectrophotometry. Statistical analysis was performed using GraphPad Prism software (version 5.0) and statistical significance was established for p-values < 0.05 in all analyses.*

***Results**: Biochemical analysis revealed significant elevations in the levels of uric acid, triglycerides, very low-density lipoprotein (VLDL), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), urea and creatinine in the Iron overload Group compared to the Non-iron overload Group and significant decreases in the high-density lipoprotein (HDL) and low-density lipoprotein (LDL). Ferritin levels correlated positively with uric acid concentrations (p-value < 0.05). The Iron overload Group showed lower interleukin-10 levels and catalase activity and higher nitrite and malondialdehyde levels compared with the Non-iron overload Group.*

***Conclusion**: The results of this study are important to develop further consistent studies that evaluate the effect of iron overload on the inflammatory profile and oxidative stress of patients with sickle cell anemia.*

***Keywords**: Anemia, sickle cell; Iron overload; Interleukin-10; Oxidative stress; Nitric oxide/metabolism*

Introduction

Sickle cell anemia (SCA) is a chronic hemolytic anemia resulting from a mutation (GAG → GTG) in the sixth codon of the β globin gene, resulting in the substitution of valine for glutamic acid and the formation of hemoglobin S (Hb S)[1,2]. It is characterized by a chronic inflammatory state with elevated levels of proinflammatory cytokines and constant cell activation, which contributes significantly to the pathogenesis of SCA[3].

Cytokines may participate in several mechanisms that promote a vaso-occlusive process, such as vascular endothelial activation, adhesion of erythrocytes and leukocytes to the vascular endothelium and platelet activation[4]. Although changes in levels of proinflammatory cytokines and anti-inflammatory properties have been previously demonstrated in SCA, the role of cytokines in SCA has not been established yet[5].

Interleukin-10 (IL-10), primarily produced by monocytes but also secreted by T and B lymphocytes when activated, is a cytokine with anti-inflammatory activity[6,7]. IL-10 suppresses inflammation by several mechanisms, including by decreasing the production of inflammatory cytokines and the toxic effects of radicals such as nitrites (NO_2^-)[8]. Few studies have evaluated the levels of IL-10 in patients with SCA and the results are still conflicting[5,9,10]. However, hydroxyurea (HU) therapy appears to increase the levels of the anti-inflammatory cytokine but the mechanism remains unclear[5].

Red blood cell transfusions have an important role in the treatment of acute and chronic complications of SCA by reducing the anemia, the concentration of Hb S and hemolysis[11]. The clinical use of this treatment has often demonstrated a significant reduction in the incidence of acute chest syndrome, painful events, stroke and the number of hospital admissions[12,13]. However, clinical and laboratory signs resulting from iron overload (IO) are observed after ten to twenty transfusions as there is no effective route of excreting iron from the organism[14].

The excess of iron in the body is associated with several diseases and increases in reactive oxygen species (ROS), thereby contributing to a hyperoxidative state of cells[15]. Free iron acts as a catalyst for oxidation with the subsequent production of superoxide and hydroxyl radicals through the Fenton and Haber Weiss reactions[16]. Thus, this current study aimed to evaluate the impact of iron overload on the IL-10 profile, biochemical parameters and oxidative stress in patients with SCA.

Conflict-of-interest disclosure:
The authors declare no competing financial interest

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Methods

Patients

A cross-sectional study was carried out of 30 adult patients of both genders with the molecular diagnosis of SCA. All were treated with hydroxyurea and followed up in the hematology service of a referral hospital in Fortaleza, Brazil. The exclusion criteria were positive serology for viral infections (hepatitis B virus, hepatitis C virus and Human immunodeficiency virus) and evidence of clinical manifestations, such as vaso-occlusive and infectious events during the three months prior to blood collection. Patients were stratified into two groups according to the presence of iron overload. One group consisted of patients with sickle cell anemia with iron overload (Iron overload Group; n = 15) and the other, patients with sickle cell anemia but without iron overload (Non-iron overload Group; n = 15). The criterion for diagnosis of the IO was serum ferritin, with two consecutive determinations of not less than 1000 ng/mL[17]. All the patients with IO were undergoing treatment with iron chelation. An informed consent form was signed by all participants and this study was approved by the Ethics Research Committee of the Universidade Federal do Ceará in accordance with Resolution 196/96 of the National Health Institute.

Laboratory methods

Molecular diagnosis of patients was based on the polymerase chain reaction (PCR) followed by digestion using the restriction enzyme *DdeI*. The IL-10 levels were determined by enzyme-linked immunoabsorbent assay (ELISA) using the OptEIA BD™ commercial kit (BD Biosciences, San Diego, CA). The biochemical profile and iron levels were determined in a Modular P800 automatic analyzer Hitachi. Plasma levels of malondialdehyde (MDA) were quantified by the reaction with thiobarbituric acid. In this reaction, two molecules of the acid react with one of MDA to form a chromophore which has maximal absorbance in the range 532-535 nm[18]. Nitrite, a metabolite of nitric oxide, was determined in plasma using Green's method, based on the diazotization reaction, which forms a chromophore with peak absorbance at 560 nm[19]. The catalase activity in erythrocytes was determined by the spectrophotometric method, whereby the decomposition of hydrogen peroxide was monitored at 240 nm in hemolysate samples collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant and diluted in a Tris-EDTA buffer[20].

Statistical Analysis

The GraphPrism (version 5.01) program was used for statistical analysis. The Kolmogorov-Smirnov test was used to check for normal distribution of the data. The results are expressed as mean ± standard deviation. Statistical differences between groups were identified using Fisher's exact and the Mann-Whitney tests. Correlation analysis was performed using the Pearson correlation test. The level of significance was set for p-values < 0.05 in all analyzes.

Results

Of the 30 patients who participated in the study, 19 (63.3%) were female and 11 (36.7%) were male. The mean age of the study population was 38.5 ± 15.6 years. Ethnicity was predominantly Mulattos (70%), followed by Blacks (30%). There were no statistically significant differences between the studied groups in respect to the demographic parameters (p-value > 0.05). The iron profile and hematological parameters are shown in Table 1. The ferritin concentration and the number of transfusions per year were significantly different between the two groups (p-value < 0.05).

Table 1 - Iron profile and hematological parameters

Parameter	Non-iron overload Group n = 15	Iron overload Group n = 15	p-value
Hemoglobin (g/dL)	8.492 ± 1.75	8.655 ± 3.21	0.87
Hematocrit (%)	24.3 ± 4.63	25.11 ± 9.05	0.77
Mean Corpuscular Volume (fl)	97.68 ± 14.86	98.54 ± 15.18	0.87
Leukocytes (x $10^3/\mu$L)	7.241 ± 2.842	6.595 ± 4.215	0.63
Platelets (x 10^3/ L)	312.4 ± 128.8	305.7 ± 170.7	0.91
Serum iron (μg/dL)	144.1 ± 61.6	154.2 ± 61.8	0.67
Total iron-binding capacity (μg/dL)	286.8 ± 60.1	249.8 ± 71.7	0.18
Labile iron content (μg/dL)	144.8 ± 46.6	116.7 ± 86.5	0.33
Total sugar iron (%)	50.79 ± 1.77	61.43 ± 21.9	0.18
Ferritin (ng/mL)	413.9 ± 245.7	2286 ± 975.2	<0.001*
Blood transfusions/year (n)	7.2 ± 2.1	2.6 ± 1.06	0.003*

Results expressed as mean ± standard deviation. Student t-test: *p-value < 0.05

An analysis of biochemical parameters revealed elevated alanine aminotransferase (ALT), lactate dehydrogenase (LDH), triglycerides, very low-density lipoprotein (VLDL), urea and creatinine levels and lower high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels in the Iron overload Group when compared to Non-iron overload Group (p-value < 0.05). There was no statistically significant difference for the parameters of total cholesterol, AST and total bilirubin or its fractions (Table 2).

The mean IL-10 value in the Iron overload Group was 41.0 ± 19.0 pg/mL and in the Non-iron overload Group it was 145.4 ± 97.3 pg/mL; this difference is statistically significant (p-value < 0.05 - Figure 1). There was a negative correlation between the IL-10 levels and ferritin levels (p-value = 0.003; r = -0.48) in the participants of this study (Figure 2).

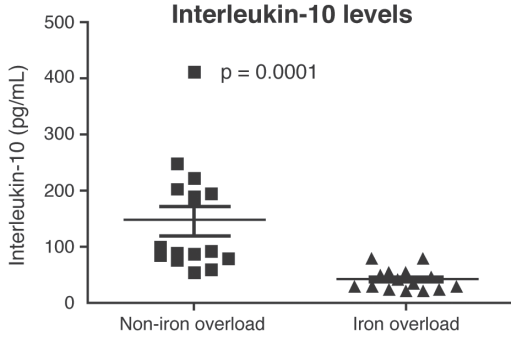

Figure 1 - IL-10 levels (n = 30)
Mann-Whitney test; *p-value < 0.05

Table 2 - Evaluation of biochemical parameters

Parameter	Non-iron overload Group n = 15	Iron overload Group n = 15	p-value
Lipid metabolism markers			
Total cholesterol (mg/dL)	107.1 ± 20.7	110.8 ± 31.5	0.71
High-density lipoprotein (mg/dL)	38.35 ± 5.3	29.09 ± 8.9	0.001*
Low-density lipoprotein (mg/dL)	66.46 ± 23.2	53.83 ± 15.9	0.04*
Very low-density lipoprotein (mg/dL)	12.88 ± 5.3	19.88 ± 10.3	0.03*
Triglycerides (mg/dL)	64.41 ± 26.8	99.38 ± 53.6	0.03*
Hepatic dysfunction and hemolysis markers			
Aspartate aminotransferase (U/L)	28.50 ± 20.8	33.55 ± 12.1	0.425
Alanine aminotransferase (U/L)	17.43 ± 5.6	23.81 ± 7.3	0.03*
Total bilirubin (mg/dL)	1.411 ± 1.3	1.164 ± 0.95	0.558
Indirect bilirubin (mg/dL)	1.084 ± 1.3	0.8012 ± 0.82	0.485
Direct bilirubin (mg/dL)	0.3273 ± 0.24	0.3627 ± 0.23	0.691
Lactate Dehydrogenase (U/L)	509.7 ± 249.5	901.7 ± 688.1	0.04*
Renal dysfunction markers			
Urea (mg/dL)	21.50 ± 10.2	40.13 ± 31.8	0.03*
Creatinine (mg/dL)	0.6634 ± 0.26	1.196 ± 0.96	0.04*

Results expressed as mean ± standard deviation. Student t-test: *p-value < 0.05

The mean uric acid level in the Iron overload Group was 6.368 ± 1.9 mg/dL, while in the Non-iron overload Group it was 4.512 ± 1.5 mg/dL (p-value = 0.0038 - Figure 3). There was a negative correlation between the IL-10 levels and uric acid levels in the study participants (p-value = 0.001; r = 0.56 - Figure 4).

The oxidative stress analysis showed that the Iron overload Group presented higher levels of nitrites and MDA and lower catalase activity compared to the Non-iron overload Group (p-value < 0.05 - Table 3). The levels of IL-10 correlated negatively with MDA levels (p-value = 0.0075; r = -0.4631) and nitrite levels (p-value = 0.0396; r = -0.3735) and positively with catalase activity (p-value = 0.0059; r = 0.4691 - Figure 5).

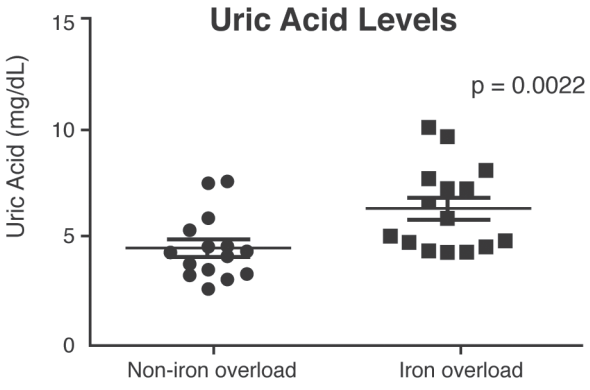

Figure 3 - Evaluation of uric acid (n=30)
Mann-Whitney test; *p-value < 0.05

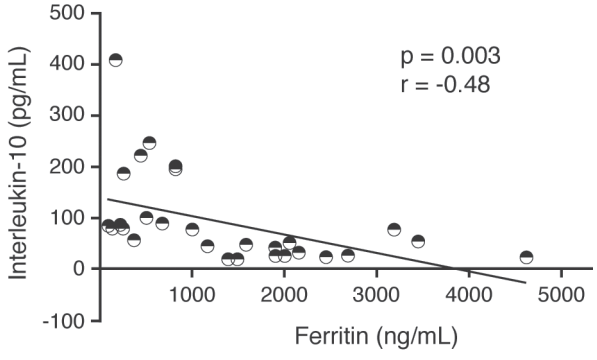

Figure 2 - Correlation between IL-10 levels and ferritin levels (n = 30)
Pearson correlation test; *p-value < 0.05

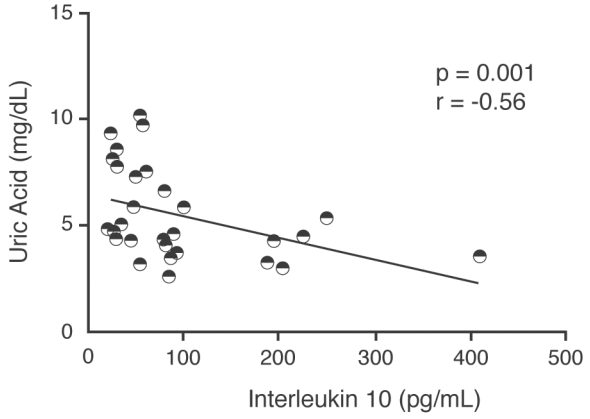

Figure 4 - Correlation between IL-10 levels and uric acid levels (n = 30)
Pearson correlation test; *p-value < 0.05

Table 3 - Oxidative stress biomarkers

Biomarker	Non-iron overload Group n = 15	Iron overload Group n = 15	p-value
Malondialdehyde (μM)	5.33 ± 2.34	8.32 ± 2.25	0.0008*
Nitrite (μM)	1.019 ± 1.13	2.513 ± 1.78	0.0003*
Catalase (U/mL)	1391 ± 322.3	982.0 ± 216	0.0002*

Results expressed as mean ± standard deviation. Student t-test: *p-value < 0.05

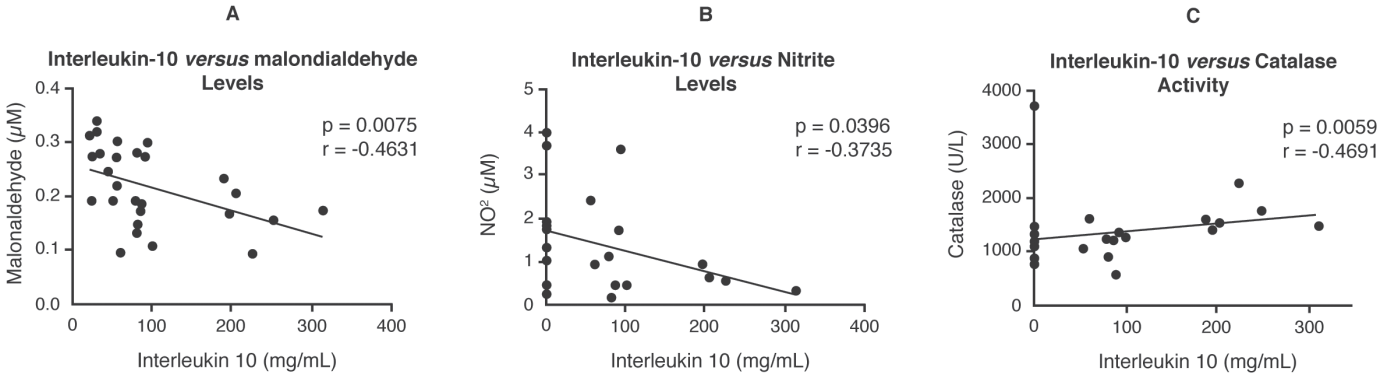

Figure 5 - Correlation between the IL-10 levels and MDA (A) and nitrite (B) levels and catalase activity (C) (n = 30)
Pearson correlation test; *p-value < 0.05

Discussion

Excess intracellular iron is potentially toxic due to catalytic reactions that favor the formation of free radicals that promote lipid peroxidation of cellular components, progressing to necrosis and/or apoptosis[21]. The results of this study revealed a significant increase in ALT, urea and creatinine levels in the Iron overload Group and is thus in accordance with the literature, which reports that an excess of iron can trigger and exacerbate liver and kidney damage in patients with SCA[22-24].

LDH has been identified as an important prognostic marker in patients with SCA at risk of developing leg ulcers, pulmonary hypertension and priapism[25,26]. In this study, the elevation of serum LDH in patients with IO suggests more severe hemolysis in these patients compared to patients without IO. The excess of free iron in the organism favors the formation of free radicals and with oxidative stress can damage the membranes of erythrocytes, exacerbating the hemolysis.

In general, SCA patients have alterations in their lipid metabolism. Hypocholesterolemia and reductions in the HDL and LDL levels are the most documented findings in these patients, however their clinical significance is not yet fully known[27]. Our data revealed significant decreases in total cholesterol, HDL and LDL levels and increases in VLDL and triglyceride levels in patients with IO, which is in agreement with literature data. This shows that oxidative stress generated by chronic hemolysis and IO may be associated with reduced plasma lipids except triglycerides. Moreover, hepatic dysfunction, which is normally present in these situations, reduces the endogenous cholesterol and amplifies the changes in the lipid profile in SCA patients[28,29].

Some studies suggest that high uric acid levels can promote organ damage and may have a deleterious effect on endothelial cell function[10]. Furthermore, uric acid can act as pro-oxidant, particularly at high concentrations[11]. These observations illustrate a potentially negative contribution of high uric acid levels in SCA. Our results confirm this statement as shown by the negative correlation between uric acid and IL-10 levels.

IL-10 is a cytokine with potent anti-inflammatory activity which reduces the production of various cytokines including IL-1, IL-6, IL-8, IL-12, TNF-α and GM-CSF to promote uptake and retention of iron in the reticuloendothelial system[7,30,31]. There are no reports in the literature on the influence of IO on the level of this cytokine in SCA. The result of the correlation between IL-10 levels and MDA, nitrite and catalase activity in our study suggest that significantly reduced IL-10 levels in the group with IO can contribute to a hyperoxidative state due to lower uptake of free iron leading to organ damage by ROS. Furthermore, the reduced IL-10 levels may be associated with elevated proinflammatory cytokine levels that are, in part, responsible for the vaso-occlusive phenomenon. The negative correlation between the IL-10 levels and ferritin levels reinforces the existence of a clear inflammatory state in patients with IO as a result of iron excess.

Several studies have demonstrated a significant increase in oxidative stress biomarkers in SCA patients compared with healthy subjects[32-35]. In SCA, oxidative stress occurs by mechanisms intrinsic to the pathophysiology of the disease, such as the increased rate of auto-oxidation associated to Hb S and reduced bioavailability of nitric oxide (NO)[36,37]. The oxidative stress analysis in patients of this study revealed a hyperoxidative status exacerbated in the group with IO by high MDA and nitrite

levels and by a reduction in the antioxidant enzyme catalase activity when compared with the group without IO. Our results are consistent with the literature, which indicates that transfusional IO is a enhancing factor of oxidative stress in SCA[38,39].

MDA is a biomarker of damage caused by ROS derived from lipid peroxidation of membranes; its accumulation changes the organization of membrane phospholipids, contributing to the process of cellular degeneration[34]. Excess iron plays an important role in the increased production of ROS, including the hydroxyl radical (OH). In addition to damage to the cell membrane and cellular components, the radical OH- reacts with NO, thereby reducing its bioavailability[33]. NO is known for its antioxidant activity. One of the mechanisms involved is to induce heme-oxidase 1 (HO1). HO-1 removes the heme in the plasma, thereby preventing its participation in reactions to form the toxic species of the organism and peroxidation, such as OH-[40]. With the increased consumption of NO in patients with IO, its antioxidant activity is compromised, thereby increasing the hyperoxidative state and inflammation present in SCA individuals. The correlations in this study between the levels of IL-10 and oxidative stress parameters support this theory.

Conclusion

The results of this study are important to develop more consistent studies that evaluate the effect of IO on the inflammatory profile and oxidative stress in SCA patients, as well as to investigate new markers able to predict organ damage with greater specificity and thus contribute to the treatment and prognosis of these patients.

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