Correlation of low levels of nitrite and high levels of fetal hemoglobin in patients with sickle cell disease at baseline

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Background: *Sickle cell disease is a hemoglobinopathy characterized by hemolytic anemia, increased susceptibility to infections and recurrent vaso-occlusive crises that reduces the quality of life of sufferers.*

Objective: *To evaluate the correlation of the levels of lactate dehydrogenase, malonaldehyde and nitrite to fetal hemoglobin in patients with sickle cell disease not under treatment with hydroxyurea in outpatients at a university hospital in Fortaleza, Ceará, Brazil.*

Methods: *Forty-four patients diagnosed with sickle cell disease were enrolled at baseline. Diagnosis was confirmed by evaluating the beta globin gene using polymerase chain reaction-restriction fragment length polymorphism. The concentration of fetal hemoglobin was obtained by high-performance liquid chromatography. Serum levels of nitrite, malonaldehyde and lactate dehydrogenase were measured by biochemical methods.*

Results: *Significantly higher levels of lactate dehydrogenase, nitrite and malonaldehyde were observed in patients with sickle cell disease compared to a control group. The study of the correlation between fetal hemoglobin levels and these variables showed a negative correlation with nitrite levels. No correlation was found between fetal hemoglobin and malonaldehyde or lactate dehydrogenase. When the study population was stratified according to fetal hemoglobin levels, a decrease in the levels of nitrite was observed with higher levels of fetal hemoglobin (p-value = 0.0415). Conclusion: The results show that, similar to fetal hemoglobin levels, the concentration of nitrite can predict the clinical course of the disease, but should not be used alone as a modulator of prognosis in patients with sickle cell disease.*

Keywords: *Lactate dehydrogenase; Malonaldehyde; Anemia, sickle cell; Nitrite*

Introduction

Sickle cell disease (SCD) is an inherited disorder of hemoglobin (Hb) synthesis, caused by a point mutation (GAG→GTG) in the beta globin gene, causing an abnormal Hb, Hb S, with consequential physical and chemical modifications of the Hb molecule[1,2]. Hb S is less soluble than Hb A, the normal Hb, when deoxygenated and is polymerized into rigid fibers that cause deformation of the erythrocytes (red blood cells) as well as the rigidity and occlusion of the microcirculation[3].

The clinical course of SCD is variable. Different factors are associated, such as the coexistence of alpha-thalassemia, the haplotypes of Hb S and Hb F levels[4]. The increased levels of Hb F are associated with reduced morbidity and mortality[5].

Excessively high levels of free Hb with its catalytic action on oxidative reactions, the chronic inflammatory state and self-oxidation of Hb S contribute to oxidative stress in SCD[6].

The disproportionately high levels of free radicals induce lipid peroxidation, with increased rigidity and altered permeability of the erythrocyte membrane. Chronic stress produces endothelial dysfunction, inflammation and damage to organs, and is associated with chronic hemolysis and complications such as leg ulcers and pulmonary hypertension[7-9].

The erythrocyte membrane may be the target of reactive oxygen species (ROS), leading to the formation of oxidized products that act as biomarkers of oxidative stress. Malonaldehyde (MDA) is an intermediate product of lipid peroxidation, which can compromise cell integrity and function[8].

During hemolysis, Hb dimers and arginase are released into the plasma; these consume nitric oxide (NO) generating inactive nitrates and L-arginine – the substrate for NO production – causing a reduction in the bioavailability of NO and contributing to the vaso-occlusive process. NO is a potent vasodilator and its reduction is associated with endothelial damage. As NO is an unstable parameter, its levels are estimated by measuring nitrite levels (NO_2^-), a product of the degradation thereof that is stable and so its measurement is sensitive[6].

The present study was aimed at correlating Hb F levels with MDA, NO_2^- and lactate dehydrogenase (LDH) in patients with SCD.

Methods

This was a cross-sectional study of 44 adult patients (15 male and 29 female, aged

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from 20 to 40 years) with molecular diagnosis of SCD; this number represents 60% of the patients treated in the hematology ward of a referral university hospital in Fortaleza, Ceará, Brazil. The patients were selected by analysis of medical records following the inclusion and exclusion criteria of the study. The study included patients with diagnosis of SCD confirmed by molecular biology, at baseline and not on hydroxyurea treatment. Determination of baseline was based on Ballas' criteria[10]: absence of painful episodes and/or intercurrent illnesses such as infections and inflammation in the four weeks preceding the study; no hospital admissions in the three days preceding the study and no blood transfusions in the four months preceding the study. The study excluded patients with infectious diseases, those with diagnosis of Hb SS not confirmed by molecular biology and those who used antioxidant vitamins. A control group comprised of 40 healthy blood donors, paired by gender and age, was formed. Informed consent was obtained from all individuals participating in the study. The project was submitted to and approved by the Research Ethics Committee of the Universidade Federal do Ceará (UFC) (Protocol #113.12.07). The group of patients with SCD was stratified according to Silva et al. regarding the levels of Hb F: ≤ 5% (n = 13), > 5 and ≤ 10% (n = 23) and Hb F > 10% (n = 8) in order to evaluate its association with the variables of the study (MDA, NO_2^- and LDH)[19].

Samples of venous blood were collected in a single session in tubes containing heparin and ethylenediaminetetraacetic acid (EDTA) anticoagulants. The heparinized plasma was isolated and stored at -80°C until analysis of the MDA, NO_2^- and LDH concentrations. Determination of Hb F levels and other hematological parameters and leukocyte DNA extraction were performed with the sample in EDTA.

The DNA was isolated from peripheral leukocytes using the whole blood DNA extraction kit. The beta S-globin gene was investigated by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[11]. The Hb F concentration was obtained through high performance liquid chromatography (HPLC). The hematological parameters were determined by means of an automated method using a Sysmex cell analyzer (Model: KX21N, Roche). The reticulocyte count was achieved by the manual method using a smear stained with brilliant cresyl blue[12].

MDA was determined based on its reaction with thiobarbituric acid (TBARS), where two molecules of TBARS react stoichiometrically with one molecule of MDA to form a pink chromophore, which has maximum absorbance in an acidic solution at 532-535 nm[13]. The NO_2^- concentration was determined using Green's method[14], which is based on identifying the presence of NO_2^- by the diazotization reaction with the formation of a pink chromophore, with a peak absorbance at 560 nm. The measurement of LDH was performed using the kinetic method whereby LDH catalyzes the reduction of pyruvate with NADH, yielding NAD^+. The catalyst concentration is determined based on the rate of decomposition of NADH, measured by the decrease in absorbance at 340 nm, according to recommendations of the manufacturer (Bioclin®, Belo Horizonte, Brazil).

Statistical analysis

The GraphPrism computer program (version 5.01) was used for statistical analysis. The Kolmogorov-Smirnov test was used to verify the normal distribution of data. Descriptive data were tabulated according to the means and standard deviation. Student's t test was performed to compare the means between the Patient and Control Groups. Statistical differences between the groups stratified according to the Hb F level were evaluated by analysis of variance (ANOVA) followed by the Tukey post test. The correlation analysis was performed by Spearman's test. The significance level defined for this study was for a p-value < 0.05 in all analyses.

Results

Demographic and laboratory characteristics of patients in the study are shown in Table 1.

The results show that not only the LDH levels but also the oxidative stress parameters (NO_2^- and MDA) were significantly higher in patients with SCD compared to the Control Group (Table 2).

Evaluation of the association of Hb F levels, stratified into three groups (Hb F < 5%, Hb F > 5 and ≤ 10% and Hb F > 10%), with the levels of LDH, NO_2^- and MDA showed that high levels of Hb F are associated with low levels of NO_2^- (p-value = 0.0415). There were no statistically significant differences for the other two parameters (Table 3).

Table 1 - Demographic and laboratory characteristics of the patients with sickle cell disease (n = 44)

Characteristic	Mean ± standard deviation
Age (years)	29.14 ± 8.9
Gender (M:F)	15:19
Hemoglobin (g/dL)	8.616 ± 1.2
Hematocrit (%)	25.29 ± 3.5
Mean corpuscular volume (fL)	95.70 ± 9.1
White blood count (x 10^3/μL)	10.7 ± 0.0312
Neutrophils (x 10^9/L)	6.201 ± 2.334
Platelets (x 10^9/μL)	403.386 ± 131.872
Fetal hemoglobin (%)	7.064 ± 5.3
Lactate dehydrogenase (U/L)	822.9 ± 38.4
Reticulocyte count (%)	9.98 ± 4.836

Table 2 - Biomarkers of oxidative stress in healthy controls and patients with sickle cell disease

	Control (n = 40)	Patients with SCD (n = 44)	p-value
MDA (μmol)	3.9 ± 3.1	17.25 ± 4.8	< 0.0001*
NO_2^- (μmol)	3.08 ± 3.6	25.63 ± 31.7	< 0.0001*
LDH (U/L)	368.2 ± 15.6	822.9 ± 38.4	< 0.0001*

Results expressed as mean ± standard deviation

MDA: malonaldehyde; NO_2^-: nitrite; LDH: Lactate dehydrogenase

*Statistically significant - Student's t-test

Table 3 - Biomarkers of oxidative stress according to the Hb F levels in patients with sickle cell disease

	Hb F ≤ 5 (n = 13)	Hb F > 5 and ≤ 10 (n = 23)	Hb F > 10 (n = 8)	p-value
MDA (µmol)	16.03 ± 4.10	17.01 ± 4.14	16.42 ± 3.5	0.6979
NO_2^- (µmol)	30,200 ± 27.27	14,900 ± 12.36	11,066 ± 6.19	0.0415*
LDH (U/L)	898,538 ± 333.30	798,066 ± 268.37	712,753 ± 85.84	0.2166

Results expressed as mean ± standard deviation
MDA: malonaldehyde; NO_2^-: nitrite; LDH: Lactate dehydrogenase
*Statistically significant - ANOVA - Tukey.

The correlation between the Hb F levels and NO_2^- in the study patients showed a significant inverse correlation (r = -0.259; p-value = 0.0425). No correlation was observed between the Hb F levels and levels of MDA, LDH and the reticulocyte count (Figure 1).

The NO_2^- levels were positively correlated with the LDH levels (r = 0.312; p-value = 0.019) and reticulocyte count (r = 0.262; p-value = 0.04 - Figure 2).

Discussion

Chronic oxidative stress contributes to endothelial dysfunction, inflammation and multiple organ damage in SCD. Recent studies indicate that roughly 50% of patients with SCD exhibit endothelial dysfunction due to membrane damage, chronic hemolysis and the reduction in bioavailable NO[15,16].

Among the modifiers of clinical severity of SCD, the Hb F concentration is considered to be the most potent genetic modifier[17]. Several studies show an association of the clinical heterogeneity of SCD with Hb F levels and the intensity of the hemolytic process[3,18,19].

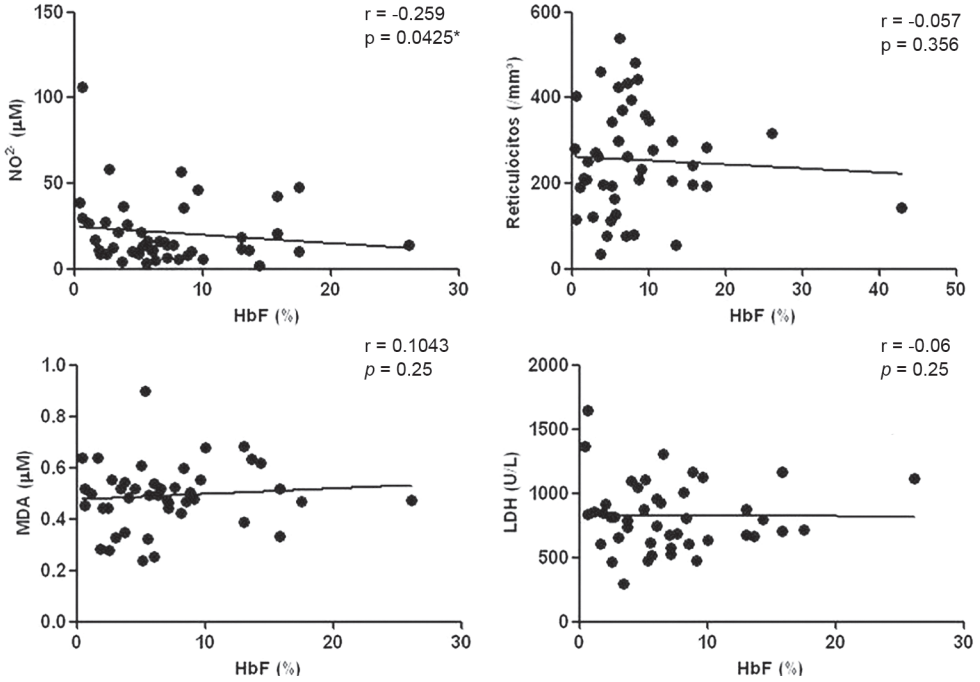

Figure 1: Analysis of the correlation between Hb F levels and levels of nitrite, lactate dehydrogenase and malonaldehyde, and reticulocyte count in patients with sickle cell disease (n = 44)
Hb F: Fetal Hemoglobin; NO_2^-: Nitrite; MDA: Malonaldehyde; LDH: Lactate dehydrogenase

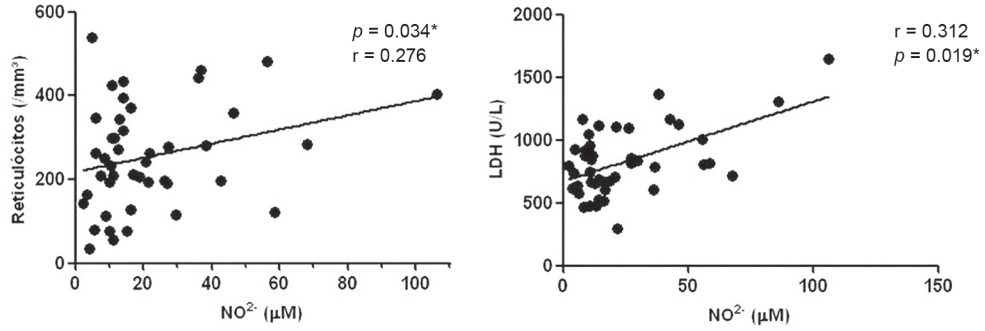

Figure 2: Correlation between the levels of nitrite and levels of LDH and reticulocyte counts in patients with sickle cell disease (n = 44).

In the present study, there was a significant increase in oxidative stress products (NO_2^-: p-value < 0.0001 and MDA: p-value < 0.0001) in adult patients with SCD compared to the Control Group. This result is consistent with several published studies that demonstrate increases in MDA and NO_2^- in patients (children, adolescents, adults) with SCD both under normal conditions and during vaso-occlusion crises; this is associated with various prognostic factors[20-25]. This result confirms that even in the absence of vaso-occlusion crises and treatment with HU, patients exhibit a hyperoxidative status and chronic hemolysis[16, 26-30].

The hematological profile of patients with SCD was characterized by mean values of Hb (8.616 ± 1.2 g/dL), Ht (25.29 ± 3.5%), mean corpuscular volume (MCV) (95.07 ± 9.1 fL), white blood cells (10.7 ± 0.0312 x 10^3/μL), neutrophils (6.201 ± 2.334 x 10^9/L), platelets (403.386 ± 131.872 x 10^9/μL), and reticulocytes (9.98 ± 4.836%), where there was moderate anemia with normal white blood counts. The reticulocyte count reflects the increase in erythropoiesis. The results are consistent with the literature[19,31]. The mean Hb F was (7.064 ± 5.3%), a result that reinforces the fact that most patients have protection against sickling[19,32,33].

A significant positive correlation was obtained between the levels of NO_2^- and the reticulocyte count and LDH level, a fact that strengthens the hypothesis that the NO_2^- in SCD may be associated with the hemolysis process. Hemolysis contributes to the formation of ROS. Oxidative stress induces lipid peroxidation and membrane instability, contributing toward an accelerated process of hemolysis[34] culminating in a more prominent bone marrow response and a consequential increase in the reticulocyte count, as SCD is a chronic hemolytic anemia[3,16].

On stratifying Hb F levels, a decrease in the levels of NO^-_2 (p-value = 0.0415) with an increase in levels of Hb F was observed. This result was consolidated by an analysis of the correlation between NO^-_2 and Hb F, where a negative correlation was found. These results support those of Salhany, who affirms that the oxy-Hb F may react with NO^-_2, leading to the formation of a higher rate of NO in relation to non-fetal cells[35]; this suggests that NO^-_2 may be being used by the Hb F for an increased production of NO. Hence, the importance of Hb F in reducing the hemolytic process and consequently in reducing the consumption of bioavailable NO remains evident, suggesting that, like Hb, NO_2^- can be used to estimate the rate of hemolysis. The results of this study corroborate those of Rusanova et al., who confirmed a protective effect of Hb F in children with SCD[26].

The MDA and LDH levels were not correlated with the Hb F levels. However, some studies have reported the importance of these parameters as laboratory markers of clinical events in SCD[6,22].

Our results reinforce the existence of hyperoxidation inherent to the disease, and that – as with Hb F levels – concentrations of NO_2^- may help predict the clinical course of the disease.

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