Study of Correlation of Nitrite Levels With Malonaldehyde and the Prognosis of Patients With Sick Cell Disease on Hydroxyurea, Ceará—BRAZIL

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Hydroxyurea (HU) is the only drug approved for the induction of fetal hemoglobin. Besides this benefit, there are others such as the reduction of leukocyte and generation of nitric oxide (NO). Sick cell anemia (SCA) is characterized by chronic hemolytic anemia and vaso-occlusive phenomena. The aim of this study was to evaluate the correlation of parameters MDA and NO₂ with the prognosis of patients with SCA as outpatients at Hospital Universitário Walter Cantídio. In all, 65 patients with SCA—51 without the use of HU (group I) and 14 chronically treated with HU (group II)—were recruited. Nitrite and malonaldehyde were determined by biochemical methods. We found that in group II there

was a significant difference of serum MDA with clinical variables: two or more transfusions during the year ($P < 0.0469$), the presence of malleolar ulcers ($P < 0.0400$), and the occurrence of vaso-occlusive episodes ($P < 0.0031$), and Group I with the occurrence of three or more vaso-occlusive episodes ($P < 0.0051$). Correlating the malonaldehyde with clinical variables in groups I and II, we observed a statistically significant relationship with two or more transfusions during the year and the presence of malleolar ulcer. Our results demonstrate that MDA levels can be used as parameter for prognosis in SCA. *J. Clin. Lab. Anal.* 25:369–373, 2011. © 2011 Wiley-Liss, Inc.

Key words: sickle cell disease; clinical profile; malonaldehyde; nitrite reductases; hydroxyurea

INTRODUCTION

Sickle cell anemia (SCA) is an inherited disorder of hemoglobin synthesis that is associated with significant morbidity and mortality due to vaso-occlusive episodes (1). Recent studies have shown that the vasculopathy in SCA is associated with endothelial damage due to the accumulation of “sickled” red blood cells, leukocytes and platelets, activation of proinflammatory substances, reduction of anticoagulant factors (antithrombin III, Protein C and S coagulation), generation of lipid peroxidation products, and increased consumption of nitric oxide (NO) (2,3).

Hydroxyurea (HU) reduces the incidence of vaso-occlusive crises in SCA and has been used to treat SCA. It has the benefit of inducing fetal hemoglobin production with concomitant increase in total hemoglobin and reduced hemolysis. In addition, HU may also be

beneficial by reducing the total count of leukocytes and the expression of adhesion molecules that contribute to vaso-occlusive processes. Some studies have shown that HU is a donor of NO, which can be oxidized by hemoglobin and form nitrosylhemoglobin and NO (4). Moreover, HU can decompose to NO chemically or enzymatically by peroxidase, urease, and catalase. Other studies have shown that HU stimulates phosphorylation and activation of endothelial NO synthase, resulting in the production of NO (5,6).

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The occurrence of vaso-occlusive episodes, especially in small vessels, is the decisive event in the pathophysiological origin of most of the signs and symptoms on clinical characteristics of patients with SCA, such as pain crises, hemolytic crises, lower limb ulcers, acute chest syndrome, splenic sequestration, priapism, aseptic necrosis of femur, retinopathy, chronic renal failure, and stroke autospplenectomy (7).

Biological macromolecules can be damaged by oxidative insult, leading to oxidized products that act as biomarkers of oxidative stress status. Oxygen radicals may provoke oxidative stress that can induce lipid peroxidation. The malonaldehyde is an intermediate product of lipid peroxidation, which is detrimental to cellular integrity and function (8).

In this context, we studied the relationship between the levels of malonaldehyde and nitrite with prognostic parameters in SCA patients with and without HU treatment.

MATERIALS AND METHODS

Setting and Design

The study design was cross-sectional and held in Fortaleza, Ceará, Brazil, in adults patients with SCA accompanied at the Hospital Universitário Walter Cantídeo outpatient clinic in the period from April 2008 to January 2009.

Material

In all, 65 patients were recruited and studied at random according to the criteria of inclusion and exclusion. Inclusion criteria were adults with a diagnosis of SCA confirmed by molecular biology with or without treatment with HU. The study excluded children and adults with SCA who have undergone transfusion therapy in the last 3 months, or who are in the use of chelating iron supplementation with vitamins C and E, or who refused to participate by not signing the informed consent.

The patients were stratified according to the use of HU, group I ($n = 51$) of patients with SCA without the use of HU and group II ($n = 14$) with the use of HU at a dosage of 30 mg/kg/day, in less than 6 months. Clinical variables were obtained from the patients' medical records.

Methodology

Samples were obtained from venous blood of each patient in a tube containing anticoagulant heparin and isolated for assay of plasma malonaldehyde, nitrite, and EDTA to confirm HbSS by PCR-RFLP (9) in patients

with SCA, and for confirmation of HbAA HPLC in individuals without hemoglobinopathies.

The method employed to determine the MDA was based on its reaction with thiobarbituric acid (TBARS) (10). The nitrite concentration was determined by Green's method (10).

Statistical Analysis

The data obtained were expressed as means $\pm$ standard deviation using the statistical software SPSS[®] 5.0. Student's *t* test was also used to compare clinical variables with the levels of MDA and nitrite. The criterion for significance was $P < 0.05$ (confidence level 95%).

Ethical Aspects

The project was submitted to the Ethics Committee of the institution where the study was conducted, and approved according to Opinion 113.12.07.

RESULTS

The prevalence of women with a mean age of 31.37 ± 11.29 years was observed in patients with SCA.

A correlation between the levels of MDA with clinical variables in patients with SCA without the use of HU, observed in group I showed a significant difference in the vaso-occlusive episodes ($P < 0.0051$) and in group II the difference was significant with two or more transfusions during the year ($P < 0.0469$), the presence of malleolar ulcer ($P < 0.0400$) and with the presence of three or more vaso-occlusions ($P < 0.0031$; Table 1). When comparing the levels of MDA with clinical variables between groups of patients with SCA with or without the use or no of HU (Groups I and II) found significant differences in two or more transfusions during the year ($P < 0.05$) and with the presence of malleolar ulcer ($P = 0.045$; Table 3).

Serum levels of nitrite in both groups showed no correlation with clinical variables (Table 2) when evaluated the groups alone or between groups I and II (Table 4).

DISCUSSION

SCA is the most common hereditary disease in Brazil and in the world (11). The phenotypic heterogeneity of the disease varies from chronic hemolytic anemia and vasculopathy with acute painful crises to multiple manifestations of specific organs with variable severity (12). Among the factors that may influence the disease process, we can highlight genetic variation, the proportion of sickle cells, endothelial activation, inflammation, oxidative stress, and hypoxia (13,14).

TABLE 1. Clinical Variables on the Level of MDA for Each Group

Clinical variables	MDA					
	Not on hydroxyurea			On hydroxyurea		
	Mean $\pm$ SD	N	P value	Mean $\pm$ SD	N	P value
<i>Transfusions per year</i>						
0–1	0.47 $\pm$ 0.14	40	>0.05	0.54 $\pm$ 0.12	10	<0.05*
≥ 2	0.52 $\pm$ 0.10	11		0.63 $\pm$ 0.04	4	
<i>Vaso-occlusion</i>						
1–2 crises	0.43 $\pm$ 0.12	24	<0.05*	0.43 $\pm$ 0.14	4	<0.05*
≥ 3	0.53 $\pm$ 0.13	27		0.6 $\pm$ 0.05	10	
<i>Leg ulcers/malleolar</i>						
No	0.47 $\pm$ 0.12	35	>0.05	0.54 $\pm$ 0.06	6	<0.05*
Yes	0.51 $\pm$ 0.15			0.64 $\pm$ 0.12		
<i>Recurrent infections</i>						
No	0.46 $\pm$ 0.15	16	>0.05	0.54 $\pm$ 0.11	8	>0.05
Yes	0.50 $\pm$ 0.11	26		0.64 $\pm$ 0.02	10	

* $P < 0.05$, statistically significant.

TABLE 2. Clinical Variables in Relation to Serum Levels of Nitrite (NO_2^-) for Each Group

Clinical variables	NO_2^-					
	Not on hydroxyurea			On hydroxyurea		
	Mean $\pm$ SD	N	P value	Mean $\pm$ SD	N	P value
<i>Transfusions per year</i>						
0–1	27.04 $\pm$ 35.18	40	>0.05	16.93 $\pm$ 12.44	10	>0.05
≥ 2	21.11 $\pm$ 16.44	11		8.35 $\pm$ 7.95	4	
<i>Vaso-occlusion</i>						
1–2 crises	28.78 $\pm$ 40.76	24	>0.05	21.86 $\pm$ 10.59	4	>0.05
≥ 3	23.10 $\pm$ 22.07	27		21.86 $\pm$ 11.31	10	
<i>Leg ulcers/malleolar</i>						
No	30.42 $\pm$ 37.41	35	>0.05	13.36 $\pm$ 11.97	6	>0.05
Yes	15.59 $\pm$ 9.20			15.32 $\pm$ 12.32		
<i>Recurrent infections</i>						
No	30.82 $\pm$ 39.29	16	>0.05	13.63 $\pm$ 11.75	8	>0.05
Yes	20.51 $\pm$ 21.72	26		19.60 $\pm$ 14.61	12	

Several studies have shown the involvement of malonaldehyde (15–17) and nitrite (18–20) in cases of SCA. The TBARS test is considered a key indicator of lipid peroxidation, which quantifies the malonaldehyde (MDA), a major breakdown product of hydroperoxides of polyunsaturated fatty acids, formed during the oxidation process (21).

When we evaluated the groups separately, we observed a significant increase in malonaldehyde in both groups of patients with SCA who had three or more vaso-occlusive crises during the year. The same occurred in relation to the levels of malonaldehyde with the variables of two or more transfusions during the year and the presence of malleolar ulcer in patients with SCA using HU. These results are relevant since they

show that the levels of malonaldehyde are directly proportional to the modulators of prognosis in SCA. In 2007, Fasola et al., evaluating the relationship between the frequency of vaso-occlusive crises and total antioxidant status (TAS), found that the average BAC in 25 patients with SCA was lower in those who had suffered three or more attacks in the previous year (16).

Correlating the malonaldehyde with clinical variables in groups I and II, we observed a statistically significant relationship with two or more transfusions during the year and the presence of malleolar ulcer, suggesting that patients with SCA using HU have a higher lipid peroxidation, requiring more blood transfusions and showing more malleolar ulcer. This may be due to the fact that patients taking HU have a more severe clinical,

TABLE 3. Comparison of Clinical Variables on the Level of MDA Between the Groups I and II

Clinical variables	Observed values				P-value
	Not on hydroxyurea		On hydroxyurea		
	MDA		MDA		
	Mean ± SD	N	Mean ± SD	N	
<i>Transfusions per year</i>					
0–1	0.47 ± 0.14	40	0.54 ± 0.12	10	>0.05
≥ 2	0.52 ± 0.10	11	0.63 ± 0.04	4	<0.05*
<i>Vaso-occlusion</i>					
1–2 crises	0.43 ± 0.12	24	0.43 ± 0.14	4	>0.05
≥ 3	0.53 ± 0.13	27	0.60 ± 0.05	10	>0.05
<i>Leg ulcers/malleolar</i>					
No	0.47 ± 0.12	35	0.54 ± 0.06	6	>0.05
Yes	0.51 ± 0.15	16	0.64 ± 0.13	8	<0.05*
<i>Recurrent infections</i>					
No	0.46 ± 0.15	26	0.53 ± 0.12	10	>0.05
Yes	0.50 ± 0.11	24	0.64 ± 0.02	3	>0.05

* $P < 0.05$, statistically significant.

TABLE 4. Comparison of Clinical Variables in Relation to Serum Levels of Nitrite (NO_2^-) Between the Groups I and II

Clinical variables	Observed values				P-value
	Not on hydroxyurea		On hydroxyurea		
	NO ₂ ⁻		NO ₂ ⁻		
	Mean±SD	N	Mean±SD	N	
<i>Transfusions per year</i>					
0–1	27.04±35.18	40	16.94±12.94	10	>0.05
≥2	21.11±16.44	11	8.35±7.95	4	>0.05
<i>Vaso-occlusion</i>					
1–2 crises	28.77±40.76	24	21.86±10.59	4	>0.05
≥3	23.09±22.07	27	11.53±11.31	10	>0.05
<i>Leg ulcers/malleolar</i>					
No	30.42±37.41	35	13.36±11.97	6	>0.05
Yes	15.59±9.19	16	15.32±12.32	8	>0.05
<i>Recurrent infections</i>					
No	30.82±39.29	26	13.63±11.75	12	>0.05
Yes	20.77±22.15	24	16.15±11.93	3	>0.05

if fitting the criteria for the use of HU, which is the presence of at least one clinical or laboratory criteria in the last 12 months. Among the clinical criteria are: three or more episodes of pain required medical hospital, acute chest syndrome, and chronic organ damage (priapism, bone necrosis, and proliferative retinopathy). Among the laboratorial criteria: concentration of Hb persistently lower than 7 g/dl and of the Hb F <8% after 2 years of age (22).

Recent studies (6,23,24) suggest that patients with SCA present a decrease in NO reserves, particularly during vaso-occlusive crises, and NO levels are inversely proportional to the painful symptoms, so the decrease in

the availability of NO may contribute to the pathophysiology of SCA (25).

The estimated levels of NO in this study were performed by measurement of serum nitrite, a breakdown product of NO that has good sensitivity and stability (17). The difficulties to measurement of nitrite result from oxidation of nitrite (within minutes) by heme proteins, such as hemoglobin. If nitrite is to be measured in plasma, blood needs to be centrifuged immediately to separate plasma from erythrocytes (26). There was no significant difference between the levels of nitrite with prognostic factors, probably due to the fact that they are not in crisis of acute hemolysis. This fact is

demonstrated by researchers (4,5,18) who showed a transient increase in NO during vaso-occlusive crises in sickle cell patients in use of HU. Morris et al. (2003) demonstrated increased levels of NO in patients with SCA in use of HU, this increase was potentiated when used in combination with arginine, but with a decline of between 3 and 4 hr (19). Lopez et al. (2003) found low levels of L-arginine and NO in adult patients with SCD during vaso-occlusive episodes when compared with SCD patients at baseline (20).

The results thus demonstrate that the levels of MDA can be used as prognostic marker in SCA.

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