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EVALUATION OF HEMATOLOGICAL VARIABLES ACCORDING TO HAPLOTYPES IN PATIENTS WITH USERS AND NONUSERS OF HYDROXYUREA IN THE SICKLE CELL ANEMIA

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ABSTRACT

Clinical and prognostic profile of patient with sickle cell disease may vary according to their type of haplotype and fetal hemoglobin level. Objective: Relate hematological variables to the haplotypes of patients with sickle cell anemia and whether or not they use hydroxyurea. **Material and methods:** Cross-sectional study of patients with sickle cell anemia users and non-users of hydroxyurea. The hematological analyzes were submitted to laboratory tests as erythrogram and reticulocyte count. The dosage of HbF was obtained by means of *High-performance liquid chromatography*. Deoxyribonucleic acid was extracted by the phenol/chloroform method for confirmation of HbS and identification of haplotypes by PCR/RFLP. **Results:** Laboratory parameters according to genotypes presented a statistically significant difference for HbF (p=0.008). The mean Hb concentration (8.9±1.1 g/dL), hematocrit (26.1±3.1%), HCM (34.4±5.0 pg) and VCM (99.9±13.5 fL) were statistically significant (p<0.001). The mean HbF level (15.6±7.4%) presented a statistically significant difference (p=0.001) in subjects taking hydroxyurea. The CAR/CAR and CAR/Ben genotypes showed high levels of HbF. **Conclusion:** Indication of the use of hydroxyurea predominates in patients with at least one CAR chromosome. Laboratory parameters Hb,VCM, HCM and HbF show better results in individuals using hydroxyurea. The CAR/CAR and CAR/Ben genotype gives higher levels of HbF.

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INTRODUCTION

Sickle cell anemia is a genetic disease, which presents clinical and hematological severity. In Brazil, based on data from the National Neonatal Screening Program (PNTN), it is estimated that 3,500 children with sickle cell disease and 200,000 with sickle cell trait are born per year (Simões *et al*., 2010).

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An evaluation study carried out in Mato Grosso do Sul, Brazil, from 2001 to 2015, showed the prevalence of sickle cell anemia followed by FSC (Kikuchi *et al*., 2018). In addition, other research conducted in quilombola communities in Tocantins also found an increase in the prevalence of sickle cell disease and sickle cell trait, specifically in the southern region (Teles *et al*., 2017). The variability of symptomatology in the clinical setting of patients with sickle cell anemia can be influenced by genetic factors including fetal hemoglobin

concentration and/or the presence of α-thalassemia, which directly affect sickle cell erythrocyte (Steinberg; Sebastiani, 2012). Patients with sickle cell anemia require therapies that markedly induce the expression of fetal hemoglobin in the erythrocyte. In this sense, hydroxyurea (HU) was the only drug approved in 1998 by the Food and Drug Administration (FDA) for use in adults with sickle cell anemia with frequent episodes of severe pain (Wong *et al*., 2014). In our previous studies involving patients undergoing HU treatment in the Mato Grosso do Sul Hospitals during four years (Bispo *et al*., 2018) and six years (Araújo *et al*., 2016), the drug induces in a marked manner the expression of HbF, improving in both cases hematological parameters, reducing episodes of severe pain and frequency of blood transfusion. The clinical picture of patients with sickle cell disease can be influenced by genetic factors. In fact, the haplotypes identified in Mato Grosso do Sul (Salles, 2018) corroborate the prevalence of CAR (Bantu) haplotypes, followed by Benin (Ben) found in other Brazilian regions (Silva *et al*., 2014, Nascimento *et al*., 2015, Watanabe, 2017).

This study was developed with the objective of evaluating the hematological variables according to haplotypes in patients with anemia user and non-user of hydroxyurea.

MATERIAL AND METHODS

A cross-sectional study with 47 people with sickle cell anemia, aged 3 to 63 years, mean age of 23 years (±12.2) treated in the Hematology Outpatient Clinic of the Hospital Maria Aparecida Pedro Pedrossian (HUMAP/UFMS) and Rosa Predrossian Regional Hospital (HRMS), in a period from December 2009 to May 2010, in Brazil. The following inclusion criteria were considered: patients diagnosed with sickle cell anemia (homozygous form) confirmed by alkaline and acid electrophoresis; who have not received blood transfusions within three months prior to sampling; medical indication for hydroxyurea therapy. Patients with interactions with other hemoglobinopathies were excluded in this study. The project was cleared by the Institution's Ethics Committee (CEP 1608) and all patients signed an Informed Consent Form. Samples were collected after each participant was informed about the study objectives and procedure. The variables studied included: Hb, red blood cells, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (HCM), mean corpuscular hemoglobin concentration (MCHC), reticulocytes, HbF and haplotypes (CAR/CAR; Atp; Ben/Ben; Ben/Atp; Ben/Cam'), in users and nonusers of hydroxyurea. The methodology for extracting deoxyribonucleic acid (DNA) to confirm HbSS and restriction polymorphism analyzes (Sutton *et al*., 1989) to characterize the types of haplotypes in sickle cell anemia in Mato Grosso do Sul has been published in a previous study (Salles, 2018).

For hematological analysis, 2 mL of peripheral blood were collected by venipuncture through vacuum collection containing ethylenediaminotretractic acid anticoagulant (EDTA-K3). Laboratory tests for erythrogram and HbF dosage were performed at the Clinical Analysis Laboratory of the University Hospital of the Federal University of Mato Grosso do Sul, Brazil. An automated method (SYSMEX-model XT-1800i™.) was used to determine red blood cell count, Hb concentration, hematocrit and hematimetric indexes. The HbF assay was performed in a High-Performance Liquid Chromatography (HPLC). For statistical analysis the Bioestat program version 5.0 was used. The statistical comparison among the different genotypes and hematological parameters was performed using the Kruskal-Wallis test followed by the post-test. In addition, the comparison between patients using hydroxyurea and those who did not used was performed using the t-student test or the Mann-Whitney test according to the normal or non-normal distribution of the data samples. The level of significance was set at 5% ($p \leq 0.05$).

RESULTS

A total of 47 participants with HbSS provided their blood samples for analysis. All patients were from Campo Grande/MS and the mean age for both sexes was 23 years (±12.2). Of the 47 participants, only 35 (74.5%) were using hydroxyurea, and 30 (63.8%) received transfusion for more than 120 days. In Table 1, the means and standard deviations of laboratory parameters among the haplotypes considered in this study showed that there was no statistically significant difference between the parameters analyzed, except for HbF ($p = 0.008$). Of the 22 CAR/CAR individuals, only 81.8% used hydroxyurea. The 14 CAR/Ben (78.6%) and seven CAR/Atp individuals (71.4%) used hydroxyurea. The comparative HbF analysis of CAR/CAR genotype individuals with CAR/Ben, CAR/Atp revealed statistically significant results. CAR/CAR individuals had a high mean HbF (16.8±8.1%). Comparing the CAR/Ben and CAR/Atp genotypes, HbF was significantly higher in the CAR/Ben genotype (12.6±7.2%) (Table 1).

Table 1. Mean and standard deviation of the hematological data of individuals with sickle cell anemia according to genotypes

Variables	CAR/CAR (n=22)	CAR/Ben (n=14)	CAR/Atp (n=7)	p
HbF (%)	[a]16.8±8.1	[a]12.6±7.2	[b]7.7±4.0	0.008
Hb (g/dL)	8.6±1.2	8.6±1.2	8.8±1.0	0.198
He (10^{12}/L)	2.5±0.4	2.6±0.5	3.0±0.8	0.210
Ht (%)	25.1±3.5	24.9±3.5	26.0±3.1	0.227
VCM (fL)	99.8±12.4	98.8±11.7	88.8±15.6	0.441
HCM (pg)	34.3±4.5	34.0±4.4	30.2±5.6	0.307
CHCM (g/dl)	34.3±0.5	34.2±0.6	33.9±0.7	0.092

Note: if $p \leq 0.05$ - statistically significant difference (different letters) - Kruskal Wallis test followed by Student Newman Keuls, calculated without the Ben/Atp and Ben/Cam haplotypes (only 1 individual each). Legend: HbF (fetal hemoglobin); Hb (Hemoglobin); He (red blood cell); Ht (hematocrit); VCM (mean corpuscular volume); HCM (Mean Corpuscular Hemoglobin); CHCM (Mean Corpuscular Hemoglobin Concentration);

On the other hand, other genotypes comparisons were not included in this analysis because a limited number of cases. In Table 2, the association between hematological data and hydroxyurea therapy showed that the mean HbF (15.6±7.4%) presented a statistically significant difference (p=0.001) in subjects who used hydroxyurea compared to those who did not use the drugHbF (6.8±5.1%).Variables, such as mean Hb concentration (8.9±1.1 g/dL), hematocrit (26.1±3.1%), HCM (34.4±5.0 pg) and VCM (99.9±13.5 fL) were statistically higher with HU therapy. Comparing the HbF values according to the haplotypes in table 3, it was observed that there was a statistically significant difference (p=0.018) among the individuals who used hydroxyurea. The CAR/CAR e CAR/Ben genotype presented a high level of HbF, with statistical significance, in relation to the CAR/Atpgenotype (Table 3). As for the HbF of those who do not use hydroxyurea it was observed that there was no significant difference between the haplotypes. The CAR/CAR individuals presented a mean (± standard deviation) of HbFof 8.9±4.6%. The Ben/Ben haplotype was identified in two individuals (one of

them adult at 46 years of age), and they had HbF less than 5% and did not use hydroxyurea.

Table 2. Mean and standard deviation of the hematological data of patients with sickle cell anemia users and nonusers of hydroxyurea

Parameters	Hydroxyurea		p
	Users (n=35)	Non-users (n=12)	
HbF (%)	15.6±7.4	6.8±5.1	(2)<0.001
Hb (g/dl)	8.9±1.1	7.5±0.9	(1)<0.001
He (10^{12}/L)	2.7±0.5	2.5±0.3	(1) 0.106
Ht (%)	26.1±3.1	22.0±2.5	(1)<0.001
VCM (fl)	99.9±13.5	89.7±5.4	(2)<0.001
HCM (pg)	34.4±5.0	30.5±1.9	(1)<0.001
CHCM (g/dl)	34.2±0.6	34.0±0.6	(2) 0.298

Note: if $p \leq 0.05$ - statistically significant difference. (1) Mann Whitney test. (2) Test t. Legend: HbF (fetal hemoglobin); Hb (Hemoglobin); He (red blood cell); Ht (hematocrit); VCM (mean corpuscular volume); HCM (Mean Corpuscular Hemoglobin); CHCM (Mean Corpuscular Hemoglobin Concentration);

Table 3. Mean and standard deviation of the hematological data of patients with sickle cell anemia according to haplotypes, users and nonusers of hydroxyurea

Haplotypes	HbF (%)	
	With hydroxyurea	Without hydroxyurea
CAR/CAR (n=22)	[a]18.5±7.7	8.9±4.6
CAR/Ben (n=14)	[a]13.9±6.8	7.9±7.9
CAR/Atp (n=7)	[b]9.4±2.5	3.6±4.5
Ben/Ben (n=2)	-	3.3±2.1
Ben/Atp (n=1)	-	8.7
Ben/Cam (n=1)	12.0	-
P	(1)0.018	(2)0.576

Note: if $p \leq 0.05$ - statistically significant difference (different letters). Kruskal Wallis test followed by Student Newman Keuls. (1) Calculated test between CAR/CAR; CAR/Ben and CAR/Atp. (2) Calculated test between CAR/CAR; CAR/Ben; CAR/Atp and Ben/Ben. Legend: CAR (Bantu or Central African Republic); Ben (Benin); Atp (Atypical); Cam (Cameroon).

DISCUSSION

In subjects with sickle cell anemia, HbF showed a marked increase in response to HU therapy for the CAR/CAR and CAR/Ben haplotypes. It should be emphasized that HbF levels in the CAR haplotype are generally associated with low concentrations (<5%) (Powars, 1991). In addition, the inheritance of at least one CAR chromosome is related to more severe clinical manifestation of sickle cell anemia (Romero; Renauld; Villalobos, 1998). A study carried out with a group of children under six with sickle cell disease in the city of Rio de Janeiro - Brazil, was the largest attendant of CAR/CAR, and the authors observed that most of the children with this haplotype had more events clinical (SILVA FILHO *et al*. 2012). The mean concentration of HbF (15.6% ±7.4), Hb (8.9±1.1), hematocrit (26.1±3.1), MCV (99.9±13.5) and HCM (34.4±5.0) presented statistically significant values in patients who use Hydroxyurea when compared to those who did not use it.

Our results were in concordance with previous studies performed in Brazil, such as the one carried out in Mato Grosso do Sul with 32 patients with HbSS, where after four years of use of HU, the following averages were obtained: HbF (14.49±7.52%), hematocrit (25.30±4.03%) hemoglobin (9.22±3.34g/dL) (Bispo *et al*., 2018). And also in Ribeirão Preto-SP, where they performed a study with 37 patients, being 26 (SS) and 11(S-beta-thalassemia) and analyzed the hematological parameters and clinical events in the previous year, and they are analyzed the results of treatment the use of HU in the first year; observed hemoglobin (8.3 g/dL for 9.0 g/dL, p=0.0003), fetal hemoglobin (HbF) (2.6% for 19.8%, p<0.0001) and VCM (89 for 105 fl, p=0.001) (Silva-Pinto *et al*., 2013). It should be argued that despite of the increase in HbF concentration in HbSS patients using HU, their distribution does not occur homogeneously in sickle cell erythrocytes. Erythrocytes with lower HbF concentration are not protected from damage caused by the polymerization. This makes the patients remain symptomatic, but with a reduced number of complications (Piel, Steinberg, Rees, 2017). The mean and standard deviation of HbF of 6.8±5.1%, found in our present study, in individuals without hydroxyurea therapy is in accordance with other results conducted in Brazil. In Rio de Janeiro, the results of Fleury (2007) obtained the mean HbF concentration of 6.66±4.61%. In Fortaleza, Silva Gonçalves and Rabenhorst (2009) found an average HbF of 6.72±3.73%. In a study done by Figueiredo *et al*. (1996) the mean HbF found was 6.6±4.1%. Furthermore, in studies conducted by Galiza Neto *et al*. (2005) it was obtained an average of 7.61±1.0% of HbF in individuals with HbSS.

To verify the variation of HbF levels according to the haplotypes, the HbF averages of the haplotypes were compared in individuals' users and nonusers of hydroxyurea. There was a statistically significant difference (p=0.018) in the individuals in therapy, whereas the CAR/CAR haplotype had a high mean HbF (18.5±7.7%). The understanding of genetics influencing the hereditary subtypes of HbSS in terms of prognosis is indispensable, since such information can aid in personalized therapy and even in the discovery of new drugs (Steinberg; Sebastiani, 2012). In summary, the lowest HbFmean (3.3±2.1%) was found in the haplotype Ben/Ben. Which coincides with a study developed in Bahia, which reported the presence of three individuals of Ben/Ben genotype with HbF less than 5% (Adorno *et al*., 2008). However, both results differ from other studies that have shown high levels of HbF in the Ben/Ben genotype (Figueiredo *et al*., 1996; Fleury, 2007; Silva *et al*., 2009). Despite the fact that there are differences between the results found in the above-mentioned studies, HU remains the therapeutic option that can benefit children and adults in reducing acute complications and neurological events of sickle cell anemia (Nevado *et al*., 1994). It is up to the patient and their family, after discussing the benefits and risks of this therapy with the hematologist to decide on the start of drug use (Wong *et al*., 2011).

Conclusions

Indication of the use of hydroxyurea predominates in patients presenting at least one CAR haplotype. The CAR/CAR and CAR/Ben genotypes showed high levels of HbF. There was no statistically significant difference between haplotypes and hematological data except for HbF. The laboratory parameters analyzed showed better results in the individuals who use hydroxyurea, demonstrating a statistically significant difference for hematocrit, Hb, VCM, HCM, HbF. There was variation in the mean levels of HbF among the different groups of genotypes with the therapeutic use of hydroxyurea.

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