EFFECT OF HYDROXYUREA ON THROMBOSPONDIN-1 AND SOLUBLE L-SELECTIN LEVELS AMONG STEADY STATE PATIENTS WITH SICKLE CELL ANAEMIA

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ABSTRACT

Background:
Sickle cell anaemia (SCA) is characterized by unpredictable episodes of vaso-occlusion. Adhesion molecules, sickled red blood cells and inflammatory stimuli all contribute to these episodes. Hydroxyurea (HU) has proven to be effective in reducing the frequency of pain episodes in SCA, notably via an Hb F dependent mechanism.

Aims and Objectives:
The objectives of this study are to determine the relationship between HU therapy and serum levels of thrombospondin-1 and soluble L-selectin in patients with SCA; and assessed the relationship between the use of HU and clinical severity of the disease.

Materials and Methods:
Sixty (60) patients with SCA (30 HU exposed and 30 HU naïve patients respectively), all in steady state, were recruited. They were administered structured questionnaire. Clinical determinants and laboratory tests, which include full blood count, soluble L-selectin and thrombospondin-1 were assessed. Clinical severity scores were also computed. Appropriate statistical tools were utilised for data analysis.

Results:
Patients with SCA exposed to HU had lower values of thrombospondin-1 and soluble L-selectin compared to HU naïve controls (P < 0.001; and P = 0.121 respectively). Most patients on HU had mild disease when compared to their HU naïve counterparts based on clinical severity score {29/30 (96.7%) vs 18/30 (60.0%), $\chi 2 = 13.918$; df = 2; P = 0.001}. There was a significant correlation between thrombospondin-1 levels and clinical severity score in both HU exposed and HU naïve patients with SCA (rho = 0.545; P < 0.001 and rho = 0.509; P = 0.002 respectively). Soluble L-selectin levels showed no significant correlation with clinical severity score in both groups (rho = 0.256; P = 0.086; and rho = 0.255; P = 0.173 respectively).

Conclusion:
The usage of HU may significantly reduce levels of thrombospondin-1, but not those of soluble L-selectin in patients with SCA.

Keywords:
sickle cell anaemia, steady state, thrombospondin-1, soluble L-selectin, hydroxyurea.

INTRODUCTION

Sickle cell anaemia (SCA) is the most severe form of a group of qualitative inherited haemoglobin disorders known as sickle cell disease (SCD).[1] Recurrent and unpredictable episodes of vaso-occlusion have been described as the hallmark of SCA.[2] Endothelial proteins involved in abnormal adhesion in patients with SCA include laminin, thrombospondin,

fibronectin, L-selectin, E-selectin, P-selectin and α5β3 integrin.[3] Hydroxyurea (HU) has been proven to effectively reduce the frequency of painful episodes in SCA.[4] This study was designed to determine the relationship between levels of adhesion molecules (thrombospondin-1 and soluble L-selectin) in hydroxyurea exposed and naïve adult patients with SCA in the steady state.

MATERIALS AND METHODS

Study design:

This was a cross-sectional study conducted at the Haematology clinic of Ahmadu Bello University Teaching Hospital (ABUTH) in Zaria, Northwestern Nigeria. G*Power 3.1.9.4 was used to conduct a power sensitivity analyses for sample size estimation.[5] This revealed that using two-tailed hypotheses and three predictors, a sample size of 60 would have an 80% power at a 0.05 alpha error probability level of detecting a medium effect size Cohen's f-square of 0.19. However, using the Departmental HU Register, only 34 patients with SCA were on HU therapy for at least one year, of whom four were lost to follow up. Therefore, 30 patients with SCA on HU and 30 HU-naïve patients with SCA as comparison were enrolled.

All patients enrolled in the study were in steady state, which was defined as a period free of crisis extending from at least three weeks since the last clinical event and three months or more since the last blood transfusion.[6] Five milliliters (5mls) of venous blood was collected from each participant and dispensed in plain and anticoagulated (EDTA) bottles. Serum was obtained from the plain bottle sample after allowing the sample to clot and then by centrifugation at 3000*g* for 10 minutes. The serum was stored at -20°C for ≤30 days before analysis. Serum was assayed for thrombospondin-1 and L-selectin levels in batches using Enzyme Linked Immunosorbent Assay (ELISA) technique (Elabscience® ELISA Kits with Lot number: E-EL-H1589 and E-CL-H0615 for thrombospondin and L-selectin respectively). EDTA blood sample was used to determine full blood count using Swelab-α Haematology analyser. Reticulocyte count was determined using manual methods.[7]

The clinical severity was determined by calculating an objective score using a method described by Okocha *et al*, [8,9] a modification of the method described by Hedo *et al*.[10]

Ethical approval:

Ethical approval was obtained from the Health Research Ethics Committee of Ahmadu Bello University Teaching Hospital. All participants signed an informed consent before enrolment. Information of participants was treated with maximal confidentiality.

Data analysis:

The data obtained was entered into the Statistical Package for the Social Sciences (SPSS) version 20.0 (2011) and analysed. Univariate analyses were conducted using means and standard deviations (SDs) for uniformly distributed variables, while median and inter-quartile ranges (IQRs) were used for non-uniformly distributed variables. Tables were utilised to present outcomes of descriptive statistics. Student T-test was used to compare means of haemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cell (WBC) and reticulocyte counts as they were normally distributed, while independent samples median test was used to compare medians. Chi square was used to determine the association between use of HU and clinical severity. Spearman correlation was used to assess the relationship between thrombospondin-1, L-selectin levels and clinical severity in HU exposed and HU naïve patients with SCA. The level of statistical significance was set at $P \leq 0.05$.

RESULTS

There were 60 patients with SCA enrolled in this study (SCA on HU = 30, SCA HU naïve = 30). The median (IQR) ages of patients with SCA on HU and HU naïve patients were 22.0 (9) years and 21.0 (10) years respectively. This difference in median age was not statistically significant using independent sample median test

(test statistic=0.267; *P*=0.796). Majority of participants were female (70%; 21/30 SCA on HU and 66.7%; 20/30 HU naïve; *P*=0.783) and unmarried (90%; 27/30 SCA on HU and 80%; 24/30 HU naïve; *P*=0.282). Patients on HU had statistically significant higher mean Hb concentrations and MCV levels when compared to HU naïve patients (8.3±0.8g/dl vs 7.3±1.1g/dl; t=4.003; *P*<0.001) and (85.1±6.4fL vs 80.0±7.9fL; t=2.738; *P*<0.001) respectively. Conversely, patients on HU had significantly lower reticulocyte, WBC and platelet counts when compared to HU naïve patients (Table 1).

Table 1: Haematological parameters, serum thrombospondin-1 and L-selectin levels of participants

Variables	Participants		Test statistic*	*P*-value
	SCA on HU (n=30) Mean ± SD	SCA HU naïve (n=30) Mean ± SD		
Hb (g/dL)	8.3 ± 0.8	7.3 ± 1.1	4.003	<0.001
MCV (fL)	85.1 ± 6.4	80.0 ± 7.9	2.738	<0.001
MCH (pg)	28.7 ± 2.5	28.8 ± 3.6	-0.147	0.883
MCHC (g/dL)	35.9 ± 1.7	35.6 ± 2.5	0.400	0.223
WBC (x10/L)	9.5 ± 2.5	12.8 ± 3.4	-4.357	<0.001
PLT (x10/L)	310.0 (194) †	424.5 (118) †	9.600 ‡	0.005
Retic (%)	10.6 ± 3.8	20.0 ± 7.5	-6.157	<0.001
Thrombospondin-1 (ng/ml)	303.2 (26.4)†	516.05 (49.1)†	60.000‡	<0.001
L-selectin (ng/ml)	1783.5 (416.0)†	1823.0 (127.0)†	3.270‡	0.121

*t-test statistics †median (IQR) ‡Independent sample median test

Table 2: Clinical severity categorization of participants (n=60)

Clinical severity	SCA on HU	SCA HU naïve
Mild disease	29 (96.7%)	18 (60.0%)
Moderate disease	1 (3.3%)	10 (33.3%)
Severe disease	0 (0.0%)	2 (6.7%)
Total	30 (100.0%)	30 (100.0%)

{$\chi 2$ = 13.918, df = 2, *P* = 0.001}

Independent samples median tests revealed that while patients on HU had statistically significant lower median (IQR) serum thrombospondin-1 levels when compared to HU naïve patients {303.2(26.4) ng/ml vs 516.1(49.1) ng/ml; test statistic=60.000; *P*<0.001}, the difference in L-selectin levels between the two groups was not statistically significant {1783.5 (416.0) ng/ml vs 1823.0 (127.0) ng/ml, test statistic=3.270; *P*=0.121; Table 1}.

Most patients on HU had mild disease when compared to their HU naïve counterparts based on clinical severity score {29/30 (96.7%) vs 18/30 (60.0%); $\chi 2 = 13.918$; df = 2; *P*= 0.001; Table 2}.

There was a positive, moderately strong and significant correlation between thrombospondin-1 levels and clinical severity score in HU exposed and HU naïve patients with SCA (rho = 0.545; *P* < 0.001; and rho = 0.509; *P* = 0.002 respectively). The levels of L-selectin, on the other hand, revealed a positive and weak but non-significant correlation with clinical severity in both groups. (rho = 0.256; *P* = 0.086; and rho = 0.255; *P* = 0.173 respectively).

DISCUSSION

The female preponderance in our study is similar to the findings of Adzika *et al* and Cobo *et al* in Ghana and Brazil respectively.[11,12] Although our sampling technique may be contributory, females have a better health seeking behaviour and are more likely to adopt key actions for health promotions, such as hospital visits, compared to men.[12] The majority of participants were single, and this is comparable to the findings of Adzika *et al* in Ghana.[12] While the average age at first marriage is 17 years nationwide, figures are lower in Northwestern Nigeria, with 48% of females married by age 15 and 78% married before their 18th birthday.[13]

This study revealed a significant increase in Hb concentration and MCV, and a significant decrease in WBC, platelet and reticulocyte counts among patients with SCA on HU, compared to HU naïve patients. This is similar to findings of Italia *et al* and Silva-Pinto *et al* in India and Brazil respectively.[14,15]The ability of HU to reduce the rate of haemolysis in SCA may be responsible for the increase in Hb levels and reduction in reticulocyte count among HU exposed patients.[16] HU-induced macrocytosis is ascribed to inhibition of DNA synthesis and is being proposed as a tool to monitor compliance.[17,18] Some studies have also ascribed usage of HU with development of megaloblastic anaemia; [19] however, this study did not investigate for the likelihood of megaloblastic anaemia among the participants. The cytoreductive ability of HU in SCA is related to its ability to inhibit DNA synthesis and by extension cell division.[17] MCH and MCHC, though slightly increased among SCA patients on HU, were not statistically significant. This differs from findings by Patel *et al* in India where SCA patients on HU therapy had a statistically significant increase in these parameters.[20] Genetic variations underlying the clinical heterogeneity of SCA such as Hb F concentration, coexisting α thalassaemia and additional polymorphisms of the β globin gene may be responsible for this difference.

The focus of most studies on HU and adhesion molecules in SCA has been to assess the expression of adhesion receptors which bind to vascular endothelium via thrombospondin and other adhesion molecules, rather than the actual levels of the adhesion molecules. These studies have led to an overall conclusion that HU therapy modulates adhesion receptor expression possibly by reducing gene expression and signalling cascade, and ultimately leading to decreased receptor activation.[21,22,23] This study revealed that HU also has the ability to reduce the actual levels of thrombospondin-1 molecules. This reduction in thrombospondin-1 levels may be explained by the ability of HU to reduce platelet count, thus reducing the quantity of alpha-granules which constitute the major reservoir of thrombospondin-1.[24]

This study noted a non-statistically significant reduction in levels of soluble L-selectin in HU exposed SCA patients. Our result corroborates the findings of Saleh *et al* who reported that HU has no demonstrable effect on soluble neutrophil adhesion molecules, L-selectin inclusive.[25] Studies have also shown that HU-exposed patients have reduced expression of L-selectin on leucocytes compared to HU naïve patients.[26] This, alongside a reduction in white blood cell count influenced by HU, contributes to low L-selectin levels.

Studies and clinical trials on usage of HU in SCA have led to a general conclusion that HU therapy can ameliorate the clinical course of SCA and modify the natural course of the disease.[27] This study observed a lower clinical severity score among HU exposed compared to HU naïve patients with SCA. Thrombospondin-1 has been described as a plasma bio-marker of disease severity in SCA from previous studies.[28] A reduction in thrombospondin-1 levels may be contributory to the reduction in disease severity noted among HU exposed patients as observed in this study. This may also explain the significant correlation between thrombospondin levels and clinical severity score of participants observed in this study.

A limitation in this study is the small sample size. However, we feel that given the low uptake of HU generally, our findings can form the bases for future large-scale studies when uptake of HU improves among patients with SCA in Nigeria. In addition, the participants in this study were not sex matched. This study did not assess genetic differences that may contribute to severity as well as expression of thrombospondin-1 and L-selectin. Baseline values of Hb F, thrombospondin-1 and L-selectin were also not determined prior to commencement of HU therapy.

CONCLUSION

This study shows that HU is associated with reduced levels of thrombospondin-1 and soluble L-selectin levels in patients with SCA. The use of HU may explain more variability in thrombospondin-1 compared to L-selectin levels in SCA patients. These findings may need to be further evaluated in future studies, because HU has multifaceted effects, all of which may contribute to clinical improvement in patients with SCA.

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Conflict of interest:
There are no conflicts of interest.

Authors' Contributions:
All authors contributed substantially to the research design, acquisition, analysis and interpretation of data, as well as drafting of the manuscript.

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