Effects of Hydroxyurea Treatment on Haemolysis in Patients with Sickle Cell Disease at Muhimbili National Hospital, Tanzania

Azra Gangji1*, Upendo Masamu2, Josephine Mgaya2, Joyce Ndunguru2,3, Agnes Jonathan2,3, Irene Kida Minja3,4, Julie Makani2,3, Emmanuel Balandya3,5, Paschal Ruggajo3,6 and Siana Nkya1,2,7,8

1Department of Biochemistry, Muhimbili University of Health and Allied Sciences (MUHAS), P. O. Box 20137, Dar es Salaam, Tanzania.
2Sickle Cell Program, Department of Hematology and Blood Transfusion, MUHAS.
3Sickle Pan-African Research Consortium (SPARCO-Tanzania).
4Department of Restorative Dentistry, MUHAS.
5Department of Physiology, MUHAS.
6Department of Internal Medicine, MUHAS.
7Department of Biological Sciences, Dar es Salaam University College of Education, Tanzania
8Tanzania Society of Human Genetics, Dar es Salaam, Tanzania.
*Corresponding author (Gangji): E-mail: azra1989mrg@gmail.com

Email addresses of co-authors: upendo.masamu@yahoo.com; jmngaya@blood.ac.tz; jndunguru@blood.ac.tz; ajonathan@blood.ac.tz; ikminja@blood.ac.tz; jmakani@blood.ac.tz; ebalandy@yahoo.com; prugajo@yahoo.com; snkyamtatiro@gmail.com
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Abstract
Tanzania is one of the countries with a high burden of sickle cell disease (SCD). Haemolytic anaemia is a clinical feature of SCD, and has been linked to major complications leading to morbidity and mortality. Treatment with hydroxyurea (HU) has shown to induce foetal haemoglobin (HbF) which in turn decreases haemolysis in patients. This study aimed to investigate the effects of HU on haemolysis in SCD patients attending Muhimbili National Hospital, Tanzania by comparing their haemolytic parameters before and after therapy. Patients meeting the criteria were initiated on HU therapy for 3 months. Two haemolytic biomarkers: unconjugated plasma bilirubin levels and absolute reticulocyte counts were measured from patients’ blood samples at baseline and after 3 months of HU therapy and compared. Both absolute reticulocyte counts and indirect plasma bilirubin levels significantly declined after HU therapy. Median (IQR) plasma unconjugated bilirubin levels dropped significantly from 20.3 (12.7–34.4) μmol/L to 14.5 (9.6–24.1) μmol/L (p < 0.001) and mean (SD) absolute reticulocyte counts dropped significantly from 0.29 (0.1) x 10^9/L to 0.17 (0.1) x 10^9/L (p < 0.001) after therapy, thus, a decline in both haemolytic biomarkers after treatment was observed. This study found a potential for use of HU therapy in managing SCD patients in our settings evidenced by improvements in their haemolytic parameters. Clinical trials with a larger sample size conducted for a longer time period would be beneficial in guiding towards the inclusion of HU in treatment protocols for the Tanzanian population.

Keywords: Sickle cell disease, hydroxyurea, haemolysis, foetal haemoglobin.
Introduction

Sickle cell disease (SCD) and thalassemia are the most common genetic disorders worldwide with 270 million carriers and 300,000 to 500,000 annual births (Weatherall et al. 2006). Up to 70% of global annual birth prevalence of SCD occurs in sub-Saharan Africa, where reports indicate that 50% to 80% of affected children die annually (WHO March of Dimes report 2006). Tanzania, is the 5th country worldwide with the highest SCD birth prevalence estimated at 11,000 births (Makani et al. 2011, Piel et al. 2013). Without intervention, it is estimated that up to 50% of children with SCD die before the age of 5 years (Weatherall et al. 2006).

SCD is a haemoglobinopathy that is largely characterized by haemolysis. The clinical manifestations of SCD result primarily from haemolytic anaemia and the effects of repeated intravascular sickling, causing vaso-occlusion and ischaemic injury (Hankins et al. 2005). Haemolytic parameters such as increased reticulocytes, an indicator of marrow compensatory response and unconjugated hyperbilirubinemia from increased catabolism (break down) of Hb guide in the diagnosis, management and monitoring of haemolysis (Barcellini and Fattizzo 2015). Lower Hb levels and higher intensity of steady-state haemolytic anaemia consistently associate with vasculopathic complications of disease, such as stroke, leg ulcers, pulmonary hypertension, priapism, and renal failure, implying that certain phenotypes of SCD relate more to haemolytic anaemia severity rather than sickle vaso-occlusion. These phenotypes result from erythrocyte injury caused by sickle haemoglobin (HbS) and its deoxygenation-induced polymerization. Erythrocyte injury leads to extra- and intravascular haemolysis, endothelial dysfunction and vasculopathy, and occlusion of small and large blood vessels, producing tissue ischaemia/reperfusion injury and inflammation (Hankins et al. 2005, Kato et al. 2007, Kato and Gladwin 2009, Nouraie et al. 2013, Kato et al. 2017).

Lower levels of foetal haemoglobin (HbF) and higher white blood cell counts are associated with an increased incidences of SCD-related events, organ damage, and mortality (Hankins et al. 2005). It is now known that higher levels of HbF diminish deoxygenated sickle globin polymerization in vitro and clinically reduce the incidence of disease morbidities (Green and Barral 2014). The rationale for use of hydroxyurea (HU), one of the few approved pharmacological therapies for SCD is based on its ability to increase foetal hemoglobin (HbF) synthesis and the inhibitory effect of HbF on polymerization of HbS (Charache 1997).

It is believed that HU influences erythropoiesis and F-cell production, which in turn determines HbF level and haemolysis and holds expanding promise for improved clinical outcomes, including decreased occurrences of pain episodes acute chest syndrome, hospitalization transfusion, and splenic autoinfarction along with improved quality of life. Prolonged use of HU sustains the clinical effects of decreased anaemia, haemolysis, and counts of white blood cells (WBCs) and platelets, in addition to the increased red cell mean corpuscular volume (MCV) (Green and Barral 2014). A recent study conducted at Muhimbili National Hospital (Osati et al. 2020) showed that the proportion of HU use by individuals with SCA at Muhimbili National Hospital was 10 per 1000, and patients on HU therapy (for at least 6 months) had increased HbF levels and showed improved clinical outcomes.
Many other observational as well as clinical studies and trials conducted on the haematological effects of HU and its effect on haemolysis (Rodgers et al. 1990, El-Hazmi et al. 1992, Jayabose et al. 1996, Scott et al. 1996, Borba et al. 2003, Zimmerman et al. 2004, Hankins et al. 2005, Yahouédéhou et al. 2019) have shown that HU decreases the rate and level of haemolysis as evidenced by decreases in bilirubin (serum/plasma), LDH, as well as reticulocyte counts, together with increasing the levels of HbF which prevents sickling and in turn reduces the rates and intensity of haemolysis as well as increasing Hb and MCV levels and decreasing WBC counts.

Recently, Tanzania through Muhimbili National Hospital, has initiated the use of HU in SCD treatment. However, there is paucity of data on evaluation of HU treatment from sub-Saharan Africa (including Tanzania), despite the fact that this is the region with a high burden of the disease and the most severe forms of SCD are prevalent. In addition, the environmental and genetic factors which affect SCD manifestations are expected to be different in different populations. Genetic variations in populations (sub-Saharan Africa versus where most studies were conducted) may translate to different clinical responses to HU, therefore justifying the need to conduct population specific studies. The recent Tanzanian guidelines for clinical management of SCD 2020 recommend use of HU in the management of SCD, however, there are difficulties with availability, accessibility and affordability of the drug. Findings from this study will not only provide data on the subject in our settings, but also show the response to HU among the Tanzanian population, and could help encourage mainstream use of HU in our settings by health care providers. Therefore, this study aimed at investigating the effects of HU therapy on haemolysis in individuals with SCD in Tanzania.

Materials and Methods
Study design
This was a nested study in a larger longitudinal study which followed up a single cohort of patients with SCD above the age of 5 years prospectively for 3 months of HU treatment. The study utilized collected blood samples from patients to measure unconjugated (indirect) plasma bilirubin levels and reticulocyte counts at baseline (before initiating HU) and after 3 months of being on a daily fixed dosage of HU. Following up patients for a 3 month period was based on references to studies conducted in a similar fashion (Rodgers et. al 1990, El-Hazmi et al. 1992). Compliance was monitored and encouraged using phone calls and/or text messages every week. The data were compared for a difference in the level of haemolysis between the two time points.

Study area
The study was conducted at Muhimbili National Hospital, Dar es Salaam, Tanzania at the haematology and paediatric clinics, whereby patients with SCD, both self-referrals as well as those referred from other health centers/hospitals in Dar es Salaam attending the clinic were recruited for the study from January to March 2020 and then followed up after three months of therapy from April to June 2020.

Study population
The study included 50 patients with SCD of the age > 5 years who were eligible for receiving HU treatment. All participants received the same dose of HU, 20 mg/kg body weight rounded off to the nearest hundred.

Inclusion and exclusion criteria
Inclusion criteria
This study included SCD patients who consented for the study with age ≥ 5 years, individuals with SCD who were on HU, patients with recent transfusion had to have HbA < 15% prior to enrollment, absolute neutrophil count > 2 x 10^9/L, platelets >100 x
10^9/L, haemoglobin level > 5.0 g/dL, and absolute reticulocytes count > 100 x 10^9/L (unless the haemoglobin level was > 8 g/dL) at baseline. For SCD patients below the age of 18, informed consent was obtained from their parents or guardians.

Exclusion criteria
This study excluded pregnant or lactating women or patients planning to get pregnant during the study period, patients unwilling to use any form of contraception throughout the period of HU administration (HU is known to be carcinogenic, mutagenic, and teratogenic in animals (Ballas et al. 2009), patients receiving chronic transfusion therapy and patients receiving a HU dose of > 20 mg/kg/day.

Sampling technique
Convenience sampling (availability sampling) of patients was employed. Data was collected from population members who were conveniently available to participate in the study. For this study, collected blood samples of patients with SCD, who met the inclusion criteria for HU treatment and were already part of the larger study (Ref. No. DA.282/298/01.C/109) for which ethical clearance had already been granted from MUHAS, were assayed.

Sample size
A total of 50 patients out of the 100 that had been enrolled in the larger study who had good compliance to the HU regimen were included in the study. Some plasma samples clotted in the laboratory and could not be included into the study, giving us a final sample size of 39 patients. The sample size was based mainly on availability of the patients from the larger study, thus limiting the generalizability and conclusiveness of our study.

Data collection and laboratory investigations
The primary outcomes of this study were the mean or median differences in unconjugated (indirect) plasma bilirubin levels, reticulocyte counts and the HbF levels between the baseline and after 3-month treatment of HU. Demographic data of the patients and haemolytic laboratory parameters such as bilirubin levels, reticulocyte counts and the HbF levels at baseline and after 3 months of HU therapy were obtained from data collected as part of the larger study. Participants were grouped as good and poor responders based on their HbF rise post HU therapy. Good responders being those whose HbF rose to twice or more from their baseline values, while poor responders were those whose HbF did not rise or rose to less than twice their baseline values. This cut-off point was decided just for this study mainly to see if there were differences in individual responses to HU by HbF levels (since the dosage was the same for all patients) and also to see if these differences would affect the levels of haemolytic parameters being measured.

Reticulocyte counts at baseline and after 3 months of consistent and compliant use of HU therapy were obtained from serial full blood counts (on about 3 mls of whole blood collected in EDTA vacutainer tubes according to the manufacturer’s instructions) which were performed using automated haematology analyser (Sysmex XT 2000i Kobe, Japan) at the Hematology Clinical and Research Laboratory, MUHAS at every monthly visit on these patients as part of the larger study. They were performed on the same day of the sample collection.

In addition, unconjugated (indirect) plasma bilirubin levels were measured by COBAS INTEGRA 400 Plus Chemistry Analyzer (Roche Diagnostics, South Africa) using 200 μL of plasma obtained from whole blood collected in EDTA vacutainer tubes according to the manufacturer’s instructions. The plasma samples for bilirubin assay had been stored at – 80 °C at the MUHAS Haematology Laboratory for around 3 months before the assay. The plasma samples (collected and stored for the parent study were utilized for our study). Plasma can be utilized to measure unbound unconjugated bilirubin levels (Ahlfors 2000). The plasma samples were assayed at the
MUHAS–Harvard Clinical Research Laboratory.

Data processing and analysis
Data were analysed using SPSS version 20. The group mean and standard deviation of absolute reticulocyte counts (being parametrically distributed), and group median and interquartile range of unconjugated (indirect) plasma bilirubin (being non-parametrically distributed) at baseline and after 3 months of HU therapy were determined. Normality assumptions were tested using Shapiro Wilk test. Both descriptive and inferential statistics were used. Comparison of group means/medians before and after therapy (these were paired samples) was performed using paired t-test for parametrically distributed data (absolute reticulocyte counts and HbF levels) and Wilcoxon signed-rank test (for matched pairs) for non-parametrically distributed paired data (unconjugated plasma bilirubin). Differences in levels of haemolyis before and after therapy (these were independent samples) were also compared among good versus poor responders using independent t-test for parametrically distributed data (absolute reticulocyte counts and HbF levels) and Wilcoxon rank-sum test for non-parametrically distributed data (unconjugated plasma bilirubin). Statistical significance was determined at a p-value of ≤ 0.05.

Ethical clearance and consideration
This was a nested study in a larger study with ethical clearance granted from MUHAS with Ref. No. DA.282/298/01.C/109. Ethical clearance was obtained from the Senate of Research and Publications Committee of the Muhimbili University of Health and Allied Sciences (MUHAS). Informed consent was obtained from patients. For SCD patients below the age of 18, informed consent was obtained from their parents or guardians. Confidentiality of the study participants was ensured using codes instead of participant’s names. Participants were given feedback on the laboratory results as well as appropriate medical counselling as needed. Refusing to participate in this study did not affect the services provided to the SCD patients attending the clinics.

Results
Demographic characteristics of the population: Age, sex and response to HU therapy
A total of 50 patients with SCD, who met the inclusion criteria of the study were recruited. Of these, 11 patients’ plasma samples could not be analysed due to clotting in the laboratory and these patients had to be excluded from the study, giving us a sample size of 39 patients (whose plasma samples were analysed successfully). The age range in this group was from seven years to sixty years of age with the median (IQR) age being 22 (17-26) years. Out of the 39 participants, 22 (56.4%) were females and 30 (77%) were good responders to HU therapy.

Description of haemolytic markers
Table 1 shows the values of haemolytic parameters at baseline and after 3 months of HU treatment, comparing them for a significant change post HU therapy.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before HU therapy</th>
<th>After HU therapy</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Mean (SD)</td>
<td></td>
</tr>
<tr>
<td>Plasma unconjugated bilirubin (μmol/L)</td>
<td>20.3 (12.7–34.4)</td>
<td>14.5 (9.6–24.1)</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Absolute reticulocyte count (x 10⁹/L)</td>
<td>0.29 (0.1)</td>
<td>0.17 (0.1)</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>Foetal haemoglobin (%)</td>
<td>4.9 (2.6)</td>
<td>13.2 (6.3)</td>
<td>&lt; 0.001**</td>
</tr>
</tbody>
</table>

* Wilcoxon signed-rank test; **Paired t-test.
Effect of HU treatment on haemolytic parameters
We observed a significant decline in both haemolytic parameters: absolute reticulocyte counts as well as plasma unconjugated bilirubin levels after 3 months of HU treatment. Average HbF levels rose significantly to about two and a half times the baseline values post HU therapy.

Response to HU therapy
Table 2 shows the average decline in haemolysis after HU therapy among good versus poor responders and compares them for significant differences.

Table 2: Differences in haemolytic parameters before and after HU therapy among good versus poor responders (n = 39)

<table>
<thead>
<tr>
<th>Average differences in haemolytic parameters after HU therapy (Before HU – After HU therapy)</th>
<th>Good Responders</th>
<th>Poor Responders</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma unconjugated bilirubin (μmol/L) [Median (IQR)]</td>
<td>5 (2.2–10.3)</td>
<td>2.9 (1.4–10.8)</td>
<td>0.9*</td>
</tr>
<tr>
<td>Absolute reticulocyte count (x 10^9/L) [Mean (SD)]</td>
<td>0.14 (0.07)</td>
<td>0.06 (0.09)</td>
<td>0.03**</td>
</tr>
</tbody>
</table>

*Wilcoxon rank-sum test  **Independent t-test.

Good versus poor responders to HU therapy
There was a significantly greater decline in mean absolute reticulocyte counts post HU therapy (p = 0.03) among the good responders as compared to the group that had poor responses to the HU treatment.

Discussion
The findings of this study showed that patients with SCD on HU treatment showed a significant decline in both haemolytic parameters (plasma unconjugated bilirubin as well as absolute reticulocyte counts). Most participants (77%) were good responders to the HU treatment. Haemolysis is a key phenomenon in SCD, and hence, it is important to monitor haemolysis related parameters following SCD interventions such as HU. Choice of the selected parameters was led by (i) the proven biological relationship between these parameters and haemolysis, (ii) available samples, and (iii) easy and accessible laboratory assays with a short turn around time.

Absolute reticulocyte counts
A decline in the mean (SD) absolute reticulocyte count was observed from 0.29 (0.1) x 10^9/L at baseline to 0.17 (0.1) x 10^9/L after 3 months of HU treatment. HU induces formation of HbF which decreases sickling of HbS, which in turn leads to a decline in premature haemolysis of red blood cells found in the SCD patients. This decreases the need for increased bone marrow erythropoiesis as a compensatory mechanism during haemolysis, thus the decline in absolute reticulocyte counts. Our findings are in line with the findings of other studies which have shown that HU lowers the reticulocyte counts in patients with SCD. A trial carried out in Saudi Arabia (El-Hazmi et al. 1992) on 21 adult patients with severe forms of SCD, treated for a similar time period (3 months) with HU therapy showed a decline in the reticulocyte counts from 13.7% before therapy to 4.09% after HU therapy (p = 0.0001). Other similar studies carried out for longer durations in the USA (Jayabose et al. 1996, Zimmerman et al. 2004, Hankins et al. 2005) and Portugal (Yahouédéhou et al. 2019) also showed a significant decline in reticulocyte counts after HU therapy. In a case-control study conducted in Brazil (Borba et al. 2003) where treatment periods varied between 7 and 72 months with an average of 26.4 months, the control group had an absolute reticulocyte count of 174.5 x 10^9/L, while the group that was on HU therapy had an absolute reticulocyte count of 74.9 x 10^9/L (p = 0.0015),
showing that the SCD patients treated with HU had significant decreases in haemolysis. Measuring of the other reticulocyte indices such as immature reticulocyte fraction (IRF) which is a good indicator of bone marrow erythropoiesis in response to hemolysis and is markedly increased in SCD (Bagdasaryan et al. 2007), and could prove beneficial in highlighting the importance of the HU on haemolysis and should be considered in future studies of the like.

**Plasma unconjugated bilirubin**

A similar trend with the plasma unconjugated bilirubin levels was observed in this study. The median (IQR) plasma unconjugated bilirubin levels dropped from 20.3 (12.7–34.4) μmol/L at baseline to 14.5 (9.6–24.1) μmol/L post HU therapy. HbF production (due to HU) decreases sickling of HbS, which in turn leads to a decline in premature haemolysis of red blood cells found in SCD patients. Decreased break down of erythrocytes leads to a fall in production of unconjugated bilirubin, the end product of haemoglobin catabolism in the liver. These findings were similar to the findings of other studies done to investigate the effects of HU in SCD patients that also showed a decline in bilirubin levels. A study conducted done for a similar time period (3 months) in the USA (Rodgers et. al 1990) on 10 SCD patients showed a statistically significant (p < 0.03) drop in serum indirect bilirubin levels, from a mean of 35.6 μmol/L before therapy to 18.8 μmol/L after the HU therapy. A similar trial also carried out for 3 months in Saudi Arabia (El-Hazmi et al. 1992) involving 21 adult patients with SCD showed a decline in total bilirubin levels (from a mean of 81.86 μmol/L to 37.10 μmol/L (p = 0.0011)) after the HU therapy. Other studies carried out in the USA (Jayabose et al. 1996, Scott et al. 1996, Zimmerman et al. 2004) for longer time periods that measured total bilirubin levels (rather than indirect bilirubin) also showed significant declines after the HU therapy, thus the decline in the rate of haemolysis.

**Good versus poor responders to HU therapy**

It was observed that the average difference in absolute reticulocyte counts before and after HU therapy was greater among the good responders (0.14 (0.07) x 10⁹/L) as compared to poor responders (0.06 (0.09) x 10⁹/L) among poor responders (p = 0.03), implying that the better the response to HU (as evidenced by a greater rise in HbF levels), the greater the improvement in haemolytic parameters, and the greater the decline in haemolysis. Greater increases in HbF could imply decreases in sickling of HbS to a greater extent leading to a greater decline in compensatory reticulocyte production, explaining the better outcomes among the good responders.

**Limitations**

The time period for which the study participants were followed (3 months) was a shorter one due to a limited time allocated to complete the study as this was a dissertation study. Although there are few studies of similar kind that have been conducted for 3 months, most of such studies are usually carried out for a much longer time, following up participants to study the effects of HU therapy in depth and detail. The small sample size of this study was based on convenient sampling (availability of SCD patients from the larger study for the allocated time), thus we would like to caution the generalizability of the study. Also, from the recruited patients (50), some of the samples that clotted in the laboratory (11) could not be utilized for analysis, decreasing the sample size further to 39. Confounding factors that could raise/decrease levels of plasma unconjugated bilirubin and absolute reticulocyte counts were not assessed.

**Conclusions and Recommendations**

In patients with SCD, HU therapy lowers indirect bilirubin levels as well as the reticulocyte counts, therefore lowering the rate of haemolysis, indicating the effectiveness of HU therapy for SCD. It was observed that even in a short period of time, there was a decrease in the rate of haemolysis, which is one of the
The authors declare that they have no competing interests.

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