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Original Article

GPx and Cu-Zn SOD Activities in Homozygous Sickle Cell Anemia: the Primary Role of Hydroxyurea

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ABSTRACT

Background: The study sought: (i) to compare the enzymatic antioxidant activity Cu-Zn superoxide dismutase (SOD) and gluthatione peroxidase (GPx) between homozygous sickle cell anemia (SCA) children receiving hydroxyurea (HU) and those not taking HU, (ii) to identify determinants of the decline in enzymatic antioxidant capacity and, (iii) to evaluate the correlation between enzymatic antioxydant markers and clinical and biological parameters of interest. **Methods:** A cross sectional study was conducted from 15thJune to 30thAugust 2014 in two hospitals of Kinshasa. History of the use of HU was taken and clinical examinations were performed. Blood samples were collected and data were analyzed using SPSS v21. **Results:** Seventy children (mean age: 9.9 ± 4.4 years of which 53% treated with HU) participated in the study. Compared with the untreated patients, those who received HU had higher rates of HbF (p= 0.004) and Hb (p= 0.002). They also had lower rate of neutrophils (p < 0.001), platelets (p= 0.070) and LDH (p= 0.012). Inverse correlation was observed between GPx activity and neutrophils rate (r = - 0.295; p= 0.023) and between Cu-Zn SOD and LDH (r = - 0.281; p= 0.023), total biluribin (r = - 0.254; p= 0.040), indirect bilirubin (r = - 0.258; p = 0.037) and creatinin (r = - 0.128; p = 0.034). The only determinant of lower activity of GPx was the fact of not receiving HU (OR: 10.71; CI 95%: 3.36-33.96; p < 0.001) whereas determinants of lower Cu-Zn SOD activity were the fact of not receiving HU (OR: 2.95; CI 95%: 1.01-8.61; p < 0.048) and having free plasmatic Hb below the median value (OR: 2.95; CI 95%: 1.01-8.61; p < 0.048).
Conclusion: In homozygous SCA children, Cu-Zn SOD and GPx activities are inversely correlated with markers of hemolysis and inflammation. The antioxidant capacity is greater in the group of patients receiving HU.

Key words: *Hydroxyurea, homozygous SCA, GPx, Cu-Zn SOD, Antioxydant activity.*

RÉSUMÉ

Contexte : L'étude avait pour objectifs: (i) de comparer l'activité antioxydante enzymatique Cu-Zn superoxyde dismutase (SOD) et la glutathion peroxydase (GPx) entre les enfants souffrant d'anémie falciforme type homozygote recevant hydroxyurée (HU) et ceux qui ne prennent pas HU, (ii) d'identifier les déterminants de la baisse de la capacité antioxydante enzymatique et, (iii) d'évaluer la corrélation entre les marqueurs enzymatiques anti-oxydantes et les paramètres cliniques et biologiques d'intérêt. **Méthodologie :** Une étude transversale a été menée du 15 Juin au 30 Août 2014 dans deux hôpitaux de Kinshasa. L'histoire de l'utilisation de HU a été retenue ; et les examens cliniques ont été réalisés. Des échantillons de sang ont été prélevés et les données ont été analysées par le logiciel SPSS v21. **Résultats :** Soixante-dix enfants (âge moyen: 9,9 ± 4,4 ans, dont 53% traités par HU) ont participé à l'étude. Par rapport aux patients non traités, ceux qui ont reçu HU avaient des taux plus élevés de HbF (p = 0,004) et Hb (p = 0,002). Ils avaient également de taux plus faible de neutrophiles (p <0,001), des plaquettes (p = 0,070) et la LDH (p = 0,012). Une corrélation inverse a été observée entre l'activité GPx et les taux de neutrophiles (r = - 0,295; p = 0,023) et entre Cu-Zn SOD et la LDH (r = - 0,281; p = 0,023), biluribin totale (r = - 0,254; p = 0,040), bilirubine indirecte (r = - 0,258; p = 0,037) et la créatinine (r = - 0,128; p = 0,034). Le seul déterminant de la baisse d'activité de la GPx était le fait de ne pas recevoir HU (OR: 10,71; IC à 95% : 3,36 à 33,96; p <0,001), alors que les déterminants de la faible activité de la SOD Cu-Zn étaient le fait de ne pas recevoir HU (OR: 2,95; IC à 95%: 1,01 à 8,61; p <0,048) et ayant de taux libre d'Hb plasmatique inférieure à la valeur médiane (OR: 2,95; IC à 95%: 1,01 à 8,61; p <0,048). **Conclusion :** Chez les enfants avec anémie falciforme type homozygote, les activités Cu-Zn SOD et GPx sont inversement corrélés avec des marqueurs d'hémolyse et de l'inflammation. La capacité antioxydante est plus élevée dans le groupe de patients recevant HU.

Mots clés : *Hydroxyurée, SCA homozygote, GPx, Cu-Zn SOD, l'activité Antioxydant.*

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INTRODUCTION

Sickle cell anemia (SCA) is emerging as an important model of oxidative stress in human pathology (1). The oxidative stress results from an imbalance between the production of reactive oxidant species (ROS) and antioxidant substances. The ROS influence the vaso-occlusive process by increasing the adhesive properties of leukocytes, erythrocytes and platelets to the endothelium (2). It constitutes a critical factor in endothelial dysfunction, inflammation and multiple organ damages.

Some therapeutic strategies that focus on decreasing ROS production, instead of increasing their neutralization, were also recently studied in vitro and in vivo (3). Arginine therapy, iron chelator desferoxamine, acatalase mimetic, exogenous NO treatment and an NF-KB inhibitor were shown to attenuate the oxidative stress (3-6). In the same way, according to many authors, hydroxyurea (HU), product frequently used in severe forms of SCA, can also limit ROS production and NO scavenging via a decrease in hemolysis (7, 8). Studies mostly conducted in Western countries, Latin America and Asia show that indeed homozygous SCA patients who are not taking HU have high levels of ROS that are unfortunatly correlated with poor prognosis of the disease (7-10). HU can be metabolized to nitric oxide, which has antioxidant properties (7). It has been also shown that HU induces glutathione peroxidase (GPx), an important anti-oxidant in sickle erythrocytes (11). However, the results of studies are sometimes contradictory. Other studies have shown that HU has both direct oxidant and indirect antioxidant properties (12). Its azide moiety can oxidize hemoglobin to methemoglobin (metHb) and nitrosyl hemoglobin (12). But, it is known that metHb might not be problematic. Although it cannot carry oxygen, it inhibits HbS polymerization with a potential benefit to reduce hemolysis and vaso-occlusion(13).

In sub-Saharan Africa, particularlyin DR Congo, despite a large number of SCA patients, very few of them are receiving HU (14). On the other hand, available data are scanty, let alone those on the alleged role of HU on oxidative stress. Complex effects of HU create a difficulty in predicting its net effect on oxydative stress and antioxidant capacity. The main objective of this study was to therefore compare the enzymatic antioxidant activity between SCA children receiving HU and those not taking the product. Secondary objectives were to identify the determinants of the decline in enzymatic antioxidant capacity in the whole group and to evaluate the correlation between enzymatic antioxydant markers and some clinical and biological parameters of interest.

MATERIALS AND METHODS

The study was done in two hospitals of Kinshasa/DR Congo specialized in the management of SCA: the Monkole hospital and the Saint-Crispin medical center. Only children with established diagnosis of homozygous SCA (isoelectric focusing method, Capillarys device) were enrolled after their legal guardians signed the informed consent. All recruited SCA patients were in a steady state attending routine follow-up during the study period (from 15th June, 2014 to 30th August, 2014). Patients aged < 2 years old or > 18 years old, those with acute illness, acute vaso-occlusive crises (VOC) or received blood transfusion within three months prior to enrollment were excluded. According to their age and their gender, HbSS Children regularly taking HU were matched with HbSS who have never taken HU. Detailed history taking, the number of severe VOC and transfusions in the previous 12 months were recorded and clinical examinations were performed. Blood samples were collected for determination of the hemoglobin (Hb) electrophoresis, percentage of HbF, full blood count, serum creatinine, total bilirubin and its fractions, lactate dehydrogenase (LDH), iron, ferritin, C reactive protein (CRP), plasma free Hb, glutathione peroxidase (GPx) and Cu-Zn Superoxidedismutase (SOD) activities. Single sample of urine specimens were collected in the morning. Urine samples containing blood (1+ or greater), white blood cells (1+ or greater) and/ or nitrites were excluded. Both a urine dipstick test and a urine albumin to creatinine ratio (ACR) measurement were performed.

Assays for Cu-Zn SOD and GPx were performed by Elisa dual method using a Stat Fax-brand device®. After collection, the serum was stored at -80°C until use. This Elisa experiment used the double-sandwich Elisa technique (15, 16). Briefly, the pre-coated antibody is human Cu-Zn SOD or GPx monoclonal antibody and the detecting antibody is polyclonal antibody with biotin labeled. Samples and biotin labeling antibody are added into Elisa plate wells and washed out with potassium phosphate buffer (PBS) or tris-buffered saline (TBS). Then avidin-peroxidase conjugates are added to ELISA wells; tetramethylbenzidine (TMB) substrate was used for coloring after reactant thoroughly washed out by PBS or TBS. TMB turns into blue in peroxidase catalytic and finally turns into yellow under the action of acid. The color depth and the testing factors in samples are positively correlated (15, 16).

The free plasmatic Hb was assayed spectrophotometrically using a Genesys 20® device. ACR was performed using the DCA Bayer analyzer® (Siemens Healthcare Diagnostics Pyt Ltd., 885 Mountain Highway, Australia), utilizing an immunoassay method.

The study protocol was approved by the Ethics Committee of Monkole hospital.

Statistical Analysis:

Analysis was done using the SPSS version 21. The results are presented as proportions, mean± standard deviation

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(SD) or as median with interquartile (IQ). In this study, the low level of enzymatic antioxidant activity was defined as value below the median of the results of GPx and Cu-Zn SOD in the whole group. Chi-square, T Student test and Mann Whitney U test were used for the comparison of groups, where appropriate. Pearson's coefficient correlations were used to determine the association between markers of enzymatic antioxidant activity and the other variables of interest. The determinants of the decline of antioxidant activity were investigated by logistic regression according to step down method. Statistical significance was determined by a p value < 0.05.

Anthropometric, hematologic and biological characteristics of patients

Data for clinical, hematologic and biological measurements are detailed in **Table 1**. Compared with the untreated patients, those who receive HU had higher rates of HbF and Hb. They also had lower levels of leukocytes, platelets and LDH. The levels of free plasmatic Hb, total bilirubin, indirect bilirubin, iron, ferritin, creatinin and ACR were not different between the two groups. The major VOC and transfusions were less frequent in patients receiving HU, but the difference did not reach statistical significance.

RESULTS

Table 1 Comparison of general characteristics of patients based on therapeutic status (treated with HU or not)

	Whole group n = 70	Treated n = 37	Untreated n = 33	p
Age, years	9.9 ± 4.4	10.1 ± 4.4	9.6 ± 4.4	0.674
Girls, %	44.3	43.2	45.5	0.522
Dose of HU, mg/kg	-	22 ±2	-	-
Duration of taking HU, years	-	3.3 ± 1.9	-	-
No major VOC last 12 months	25 (36)	16 (43)	9 (27)	0.253
No splenomegaly	36 (51)	27 (73)	9 (27)	<0.001
Hb, g/dl	8.2±1.3	8.7 ± 1.3	7.7 ± 1.3	0.002
Leucocytes, elts/mm^3	11618 ± 4621	9547 ± 4085	13891 ± 4121	< 0.001
Neutrophils, elts/mm^3	4640 ± 2183	3398 ± 1723	5927 ± 1850	<0.001
Platelets, elts/mm^3	380727±154522	345.030 ± 162.760	414.781 ± 141.119	0.070
Reticulocytes,%	12.0 (8.5 – 17.6)	11,0 (7,0 – 15,5)	14,0 (9,6 –19,8)	0.117
HbF, %	8.7 (3.5 – 16.9)	13.9 (6.9 – 21.4)	6.2 (2.1 – 9.7)	0.004
Indirect Bilirubin, mg/dl	1.9 (0.9 – 3.5)	1.5 (0.9 – 3.8)	1.9 (1.1 – 3.5)	0.941
Total Bilirubin, mg/dl	2.5 (1.5 – 4.0)	2.2 (1.3 – 4.3)	2.6 (1.7 – 4.0)	0.632
Creatinin, mg/dl	0.38 ± 1.05	0.38 ± 0.12	0.37 ± 0.11	0.600
GFR, ml/min/1.73 m^2	211 ± 54	209±67	215±35	0.691
LDH, UI/l	544 (400 – 771)	457 (338 – 761)	653 (490 – 788)	0.012
CPR, mg/l	3.4 (2.0– 5.1)	3.0 (1.3 – 4.5)	3.9 (2.8 – 6.9)	0.151
Iron, µmol/l	16.2 (13.2 – 19.2)	16.3 (14.0 – 21.1)	15.5 (11.5 – 17.8)	0.892
Ferritine, ng/ml	209 (106 – 453)	301 (111 – 613)	184 (104 – 378)	0.396
Free plasmatic Hb, mg/l	168 (116 – 267)	140 (120 – 240)	210 (106 – 288)	0.278
ACR, mg/g	10 (9 – 15)	10 (9 – 13)	12 (8 – 12)	0.219

Values are presented as proportions, means± SD or median with IQ. ACR: albumin creatinin ratio; CRP: C reactive protein; GFR: glomerular filtration rate; Hb: hemoglobin; HbF: fetal Hemoglobin; LDH: lactate deshydogenase; VOC: vaso occlusive crisis.

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Correlations between markers of enzymatic antioxidant activity and hematologic and biological parameters

The relationship between GPx and other parameters studied and that between Cu-Zn SOD and these parameters follows the same trend (**Table 2**). Inverse significant correlation was observed between GPx activity and neutrophils rate. A statistically significant inverse correlation was also observed between SOD and some markers of hemolysis (LDH, total biluribin, indirect bilirubin) and creatinin. The percentage of HbF was not statistically correlated to the activity of Cu-Zn SOD nor GPx.

Table 2 Correlations between GPx, Cu-Zn SOD and other parameters of interest

	GPx		Cu-Zn SOD	
	r coefficient	p value	r coefficient	p value
Hb	0.152	0.233	0.126	0.321
Leucocytes	- 0.199	0.118	- 0.086	0.501
Neutrophils	- 0.295	0.023	- 0.099	0.454
Platelets	- 0.085	0.510	- 0.039	0.763
Reticulocyte (%)	- 0.007	0.955	0.003	0.980
HbF, %	0.215	0.093	0.178	0.162
Indirect bilirubin, mg/dl	- 0.082	0.515	- 0.258	0.037
Total bilirubin, mg/dl	- 0.097	0.441	- 0.254	0.040
creatinine, mg/dl	- 0.101	0.422	- 0.128	0.034
GFR, ml/min/1.73 m^2	- 0.100	0.444	- 0.103	0.427
LDH, UI/l	- 0.169	0.182	- 0.281	0.023
CPR, mg/l	- 0.147	0.247	- 0.136	0.279
Iron, µmol/l	- 0.170	0.176	- 0.149	0.233
Ferritine, ng/ml	- 0.041	0.743	- 0.046	0.714
Free plasmatic Hb, mg/l	- 0.136	0.287	- 0.241	0.056
ACR, mg/g	- 0.165	0.268	- 0.076	0.609

ACR: albumin creatinin ratio; CRP: C reactive protein; GFR: glomerular filtration rate; Hb: hemoglobin; HbF: fetal Hemoglobin; LDH: lactate deshydogenase; VOC: vaso occlusive crisis.

Enzymatic antioxidant activity and their determinants

Cu-Zn SOD and GPx activities showed significant variability between patients (**Fig 1**). In the whole group, the median activity of Cu-Zn SOD was 229 pg/ml (IQ: 78 - 641 pg/ml) vs GPx median activity which was 220 micromol/l (IQ: 101 - 466 micromol/l).

Table 3 shows that SCA children receiving HU had greater activity of GPx while the difference of Cu-Zn SOD activity between the two groups was not statistically significant. Considering the median value of results in the whole group, the logistic regression analysis identified some factors as the determinants of the decline of enzymatic antioxidant activity. The single factor associated with lower activity of GPx was the fact of not receiving HU (OR: 10.714; CI 95%: 3.363-33.959; $p < 0.001$; $\beta=2.372$). The factors associated with lower Cu-Zn SOD activity were the fact of not receiving the HU(OR: 2.946; CI 95%: 1.008-8.610; $p < 0.048$; $\beta=1.080$) and the free plasmatic Hb below the median value (OR: 2.946; CI 95%: 1.008-8.610; $p < 0.048$; $\beta=1.080$). Variables introduced into the mathematical model included: %HbF, background of hand foot syndrom in nearly childhood, the treatment with HU, the penicillin and folic acid treatment, the plasmatic ferritin level higher than 500 ng/ml, serum iron level higher than 30 micromol/l, level of LDH higher than the median value in the whole group, microalbuminuria and the frequency of infections more than 3 during the year.

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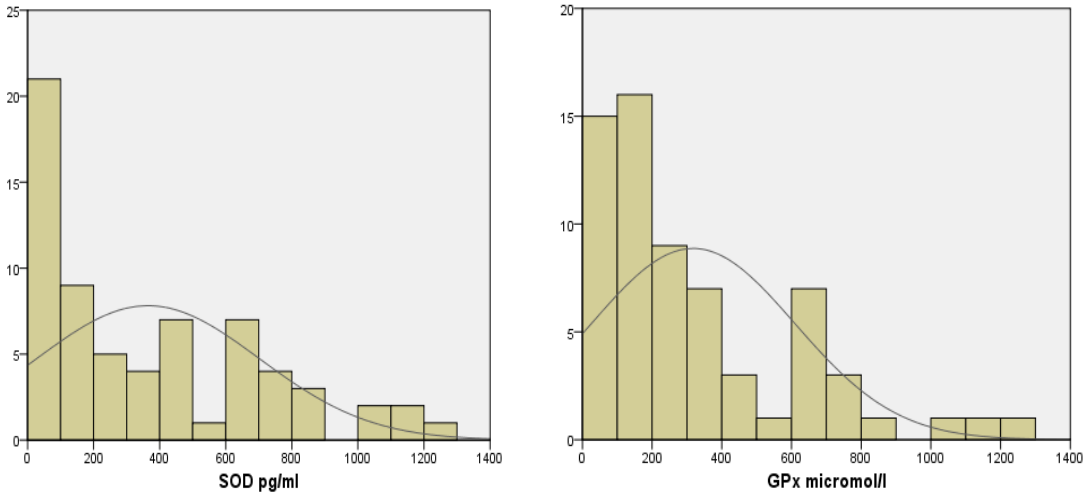

Fig 1. Distribution of enzymatic antioxidant activities in the whole group

Table 3 Comparison of enzymatic antioxidant activities based on the therapeutic status

	Treated n = 37	**Untreated** n = 33	**P**
GPx, micromol/l	391 (199 – 678)	108 (65 – 220)	< 0.001
Cu-Zn SOD, pg/ml	402 (96 – 694)	132 (53 – 407)	0.139

DISCUSSION

The present study showed that HU is associated with a better clinical and biological profile as well as to better GPx and Cu-Zn SOD activities. In the whole group, markers of hemolysis and rate of neutrophils were negatively correlated with antioxidant activity.

Many studies worldwide have shown the benefit of HU in SCA to reduce the symptoms (17-19). Our results confirm data from the literature. The precise mechanism by which HU produces its varied effects is not fully elucidated. The efficacy of HU is generally attributed to its ability to boost the levels of HbF (20). Assays have demonstrated that its target is the enzyme ribonucleotide reductase, with HU acting as a free radical that is specific for the tyrosyl groups of this enzyme. Ribonucleotide reductase is essential for deoxyribonucleic acid (DNA) synthesis, and its inhibition by HU results in S-phase cell cycle arrest. Other mechanisms may be responsible for the fact that this drug acts as a radiation sensitizer, inhibiting the repair of damaged DNA (9).

Many benefits of HU have long suggested its possible role of oxidative stress in addition to the elevation of HbF as repeatedly demonstrated in large studies (7-11). While many studies have shown that naïve SCA had higher levels of free radicals compared to patients receiving the product, studies related to the antioxidant activity are rare. In multivariate analysis, this work shows that the fact of not receiving HU multiplies by 10 times the risk of decreased activity of GPx and by 3 times the risk of decreased activity of Cu-Zn SOD. It is known that SOD are enzymes that alternately catalyze the dismutation of the superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). H_2O_2 is also damaging, but in less magnitude, and is degraded by other enzymes such as GPx and catalase (CAT). Silva *et al.*, in a study where the sample was still small, have demonstrated that in SCA, HU contributes to higher CAT

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activity and E vitamin equivalent antioxidant capacity, and to a lower lipid peroxidation (9). Torres *et al.* demonstrated that the influence of HbS on the oxidative status is reflected by increased lipid peroxidation and antioxidant status in SCA patients (21). HU improve the antioxidant capacity through several mechanisms such as its anti-inflammatory effects, the decrease in blood viscosity, increased bioavailability of NO, decreased white blood cells and adhesion molecules (7-12, 17, 18, 22).

The inverse correlation between the antioxidant enzyme activity and markers of hemolysis and neutrophils rate corroborates the literature data (23, 24). Oxidative stress is believed to aggravate the symptoms of many diseases, including SCA. When cells experience oxidative stress, ROS, which are generated in excess, may oxidize proteins, lipids and DNA - leading to cell death and organ damage (25).

Although oxidative stress is not the primary etiology of SCA, oxidative damage to the erythroid cells plays a crucial role in hemolysis due to ineffective erythropoiesis in the bone marrow and short life of red blood cells in the blood storm (25). Hemolysis, in turn, also exacerbates the oxidative stress. Indeed, when hemoglobin escapes into plasma, it can scavenge NO. The free plasmatic hemoglobin promotes highly oxidative reactions involving iron, the heme porphyrin ring and oxygen radicals that induce endothelial dysfunction (26).

The inverse relationship between the activity of GPx and neutrophil rate is explained by the fact that oxidative stress is correlated with inflammation (27). The inflammatory reaction proceeds through various phases during which neutrophils play an important role. The term inflammatory mediators is very general in so far as it includes both cytokines, endotoxin, prostaglandins and leukotrienes, histamine, microcrystals and ROS. If the ROS production is too high so that the defense systems are overwhelmed, cells are exposed to an oxidative stress which maintains the inflammatory state (27).

Ex vivo and in vitro observations implicate SOD as a mediator of renal injury (28, 29). Few years ago, Vaziri *et al.* showed that renal failure is associated with depressed SOD that can contribute to oxidative stress by increasing superoxide (30). We believe it is necessary to conduct larger studies, preferably of prospective kind in order to reach definitive conclusions.

Apart from the sample size that was small in this study, one of the limitations of this work was the fact that we did not assess the total antioxidant activity (31). Indeed, micronutrients, trace elements as well as other genetic factors are involved in the fight against ROS. The SOD was chosen because this enzyme is responsible for removing the superoxide anion, the first toxic species formed from oxygen. It thus provides the first line of defense against oxidative stress. However SOD needs trace elements such as copper and zinc (Cu-Zn SOD present in the cytosol), or manganese (Mn SOD present in mitochondria) to function properly. Low values of SOD in some patients of this study can be explained by low levels of trace elements. On the other hand, against the oxidative stress, SOD behaves in two different ways. Initially, the body will react in a moderate oxidative stress by over-expressing SOD whereas if the stress persists and produces massively toxic ROS, SOD will be destroyed and its concentration drops (32). All these considerations may partly explain the variability of results in the group of patients studied. GPx, a key enzyme for removing lipid peroxides resulting from the effect of oxidative stress on the polyunsaturated fatty acids, also needs selenium to function properly (32, 33). As SOD, glutahion seleno-dependent peroxidase behaves in two different ways against the oxidative stress: over expression of the enzyme in a first stage and then destruction if oxidative stress persists permanently (33).

CONCLUSION

The present study showed that in homozygous SCA children, the enzymatic antioxidant activity is inversely correlated with markers of hemolysis and inflammation. Cu-Zn SOD and GPx antioxidant activities are greater in the group of patients who received HU than the group not receiving it.

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Conflict of Interest

Authors declare that they have no competing interest.

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