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The Effect of Hydroxyurea on the Expression of White Blood Cell Adhesion Molecules in Patients with Sickle Cell Disease.

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ABSTRACT

Sickle cell disease is an autosomal recessive disorder that appears as a serious chronic hemolytic anemia. Vaso-occlusive crisis is the most important cause of morbidity and mortality in these patients. Recent findings showed that the expression of leukocyte adhesion molecules on vascular endothelium increased the risk of vaso-occlusive crisis in these patients. This study was conducted to identify adhesion molecules mainly involved in vaso-occlusive crisis and the effect of hydroxyurea on the expression of leukocyte adhesion molecules in patients with sickle cell disease. In this analytical study, patients were divided into two groups. In this regard, 21 patients who suffered sickle cell disease and received hydroxyurea, and 21 patients who suffered sickle cell disease but did not receive hydroxyurea. The control group comprised 21 healthy people. A blood samples was drawn from all participants, and a CBC test was performed to determine WBC count and percentage of neutrophils, lymphocytes, monocytes, and eosinophils. After Lysis of red blood cells by lysine, blood samples were stained with four types of monoclonal antibodies against surface antigens of CD11a, CD11b, CD18, and CD62L and analyzed using flow cytometry. Finally, the expressions of these markers on leukocytes were recorded as percent values. The indexes used for the three groups were compared to one another using SPSS software, ANOVA, and Tukey test. WBC count in patients was significantly higher than that in the control group. However, after using hydroxyurea, it decreased significantly in patients (*P*- value≤ 0.05). Percentage of neutrophils was high in patients, but it decreased significantly after using hydroxyurea. The analysis by flow cytometry showed that the expression of CD11a, CD11b, CD18, and CD62L markers on leukocytes increased in patients compared to that in the control group. The expression of these markers in patients with sickle cell disease significantly decreased after using hydroxyurea (*P*-value≤ 0.05). The expression of leukocyte adhesion molecules increased in patients with sickle cell disease compared to that in normal participants. As leukocytosis and increased expression of adhesion molecules in patients with sickle cell disease decreased significantly after using hydroxyurea, vso-occlusive crisis can be prevented by the use of hydroxyurea as an important strategy in pharmacological approaches.

Keywords: hydroxyurea, WBC, Sickle cell.

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INTRODUCTION

Sickle cell disease (SCD) is an autosomal recessive disorder that appears as a serious chronic hemolytic anemia. The disease is caused by a point mutation in beta-globin gene, and this mutation leads to replacement of glutamic acid with valine in the sixth amino acid position of the beta chain and produces hemoglobin S (HbS) [1]. HbS polymerization damages RBC membranes and disrupts cellular cation homeostasis that results in loss of potassium, water, and dehydration of cells, and finally, erythrocytes become irreversibly sickle. Sickle cells are tougher than the normal cells and are less flexible [2]. The toughness and deformity lead to chronic hemolysis, loss of splenic function, recurrent infections, and vaso-occlusive crisis (VOC) [3].

The VOCs are disabling attacks of abdominal, bone, and joint pain along with fever, which may be caused by masses of stickle cells stuck in small blood vessels. This occlusion results in ischemia and infarction of tissues [4]. The VOC is the most important cause of morbidity and mortality in patients with SCD [5]. It is a complex process in which different factors are involved, including erythroid and leukocyte adhesion molecules, inflammation, endothelial damage, activation of platelets and coagulation process, and the reduction of nitric oxide [6,7].

Leukocytes or white blood cells (WBC) play an important role in incidence and exacerbation of VOC because WBCs are larger than erythrocytes and less flexible, so that, they can adhere to the endothelium, especially in small vessels, and disrupt RBC and WBC movement, and consequently, increase risk of VOC [8,9]. Studies show that the expression of leukocyte adhesion molecules increased in patients with SCD compared to that in normal participants, and neutrophils in patients with SCD had greater affinity for binding to endothelial and fibronectin [10] The adhesion of leukocytes to the endothelium and other cells is mediated by adhesion molecules, such as $\alpha M\beta 2$(CD11b/CD18), $\alpha L\beta 2$(CD11a/CD18), CD62L, CD162, and CD64, binding to ICAM-1, VCAM-1, and ICAM-4 ligands on the endothelial and other cells [11].

Today, hydroxyurea (HU) is one of best drugs used for treatment of patients with SCD and has reduced mortality of these patients by 40% [12,13]. HU disrupts the cellular cycle through inhibition of ribonucleotide reductase [14]. However, molecular target of HU and the way of improving its clinical symptoms are not known completely [15]. It was first supposed that HU increased hemoglobin F, but then, it was observed that the clinical improvement happened before the increased hemoglobin F. Therefore, it was proposed that HU probably worked through other mechanisms, such as reducing leukocyte and erythroid adhesion to the endothelium and components of extracellular matrix [16]. Given the foregoing, this study was conducted to examine the effect of HU on the expression of adhesion markers mainly involved in VOC on leukocytes of peripheral blood in patients with SCD. In this regard, the involved adhesion molecules and the effect of HU were identified in order to introduce new therapeutic targets and strategies in pharmacological approaches for patients with SCD.

METHODS

This analytical study was performed on patients who suffered SCD and were being treated by oncologists in Shafa Hospital in Ahvaz, Iran. The patients with fever and infection at the time of blood sampling were excluded. The patients were divided into two groups. The first group included 21 patients who suffered SCD and received HU (these patients received HU at least 6 months before blood sampling), and the second group consisted of 21 patients who suffered SCD but did not received HU. The control group comprised 21 normal people. Each group included 11 females and 10 males. Mean age of the normal participants and patients was respectively, 31.57±1.74 and 29.73±1.81 years.

The normal participants were selected regarding blood parameters in CBC test and clinical signs. A blood sample of 5 cc was drawn from all participants and added to the tube containing EDTA anticoagulant. Firstly, the standard CBC test was performed on the peripheral blood according to the routine laboratory instructions. Then, erythrocytes were lysated using lysine (Dako Co., Danmark) on the other part of blood, and WBCs were stained with monoclonal antibodies against surface markers conjugated with fluorescent materials, including CD11a, CD11b, CD18, and CD62L (eBioscience Co., USA). The stained WBCs were analyzed using flow cytometry (*Partec,* Germany), and the expression of markers on leukocytes was recorded as percent values. Mean and standard error of the mean (Mean±SEM) of WBC count, percentage of leukocyte subgroups, and percentage of each leukocyte surface marker in the control and case groups were determined. The results

obtained from the control and case groups were analyzed using SPSS16 software and ANOVA test. Furthermore, Tukey test was used for pairwise comparison of variables. The significance level of the tests was 0.05.

RESULTS

Analysis of the results showed that WBC count in patients was significantly higher than that in the control group. However, after using HU, it decreased significantly in patients (Table 1). As shown in Table 2, percentage of neutrophils was high in patients, but it decreased significantly after using HU (*P*-value≤0.05). Although the percentage of lymphocytes decreased in the two case groups compared to that in the control group, the difference was not significant. The percentage of eosinophils and monocytes increased in the case groups compared to that in the control group, but the difference was not significant (*P*-value>0.05) **.**

According to the results of flow cytometry analysis in Table 3 and Figure 1, percentage of CD11a marker on leukocytes increased in patients compared to that in the control group. This increase was significantly reduced after using HU (*P*-value≤0.05).

The percentage of CD11b marker on leukocytes increased in patients compared to that in the control group, but it decreased significantly after using HU. The increased CD18 marker in patients was significantly reduced after using HU. The upward trend and the subsequent downward trend after medication were true also for the CD62L marker (*P*-value≤0.05).

Table 1: The comparison of mean and standard error mean (SEM) of white blood cell numbers among healthy individuals (Control group), sickle cell disease patients (SCD group) and sickle cell disease patients using hydroxyurea (SCD-HU group).

P-value	SCD-HU group (Mean±SEM)	SCD group (Mean±SEM)	Control group (Mean±SEM	white blood cell (No.)
0.018	8177 ± 800	11410 ± 1400	7490 ±280	WBC

Table 2: The comparison of mean and standard error mean (SEM) of leukocyte subsets among healthy individuals (Control group), sickle cell disease patients (SCD group) and sickle cell disease patients using hydroxyurea (SCD-HU group) according to percent (%).

P- vlaue	SCD-HU group (Mean±SEM)	SCD group (Mean±SEM)	Control group (Mean±SEM)	Leukocyte subsets
0.005	60.14 ± 1.86	70.19 ± 2.50	65.86 ± 2.08	Neutrophils
0.605	31.15 ± 2.35	30.47 ± 2.06	33.48 ± 2.12	Lymphocytes
0.068	2.250 ± 0.393	2.850 ± 0.254	1.875 ± 0.239	Monocytes
0.225	1.692 ± 0.286	2.105 ± 0.215	1.545 ± 0.207	Eusinophils

Table 3: The comparison of mean (M) and standard error mean (SEM) of leukocyte expression of different markers among healthy individuals (Control group), sickle cell disease patients (SCD group) and sickle cell disease patients using hydroxyurea (SCD-HU group) according to percent (%).

P- vlaue	SCD-HU group (Mean±SEM)	SCD group (Mean±SEM)	Control group (Mean±SEM)	Leukocyte expression of markers
0.023	25.14 ± 1.91	33.78 ± 2.65	29.38 ± 1.84	CD11a
0.003	21.96 ± 2.41	32.27 ± 2.47	24.63 ± 1.24	CD11b
0.008	42.31 ± 1.92	51.10 ± 2.42	44.54 ± 1.51	CD18
0.018	23.06 ± 1.33	27.55 ± 2.07	21.49 ± 0.967	CD62L

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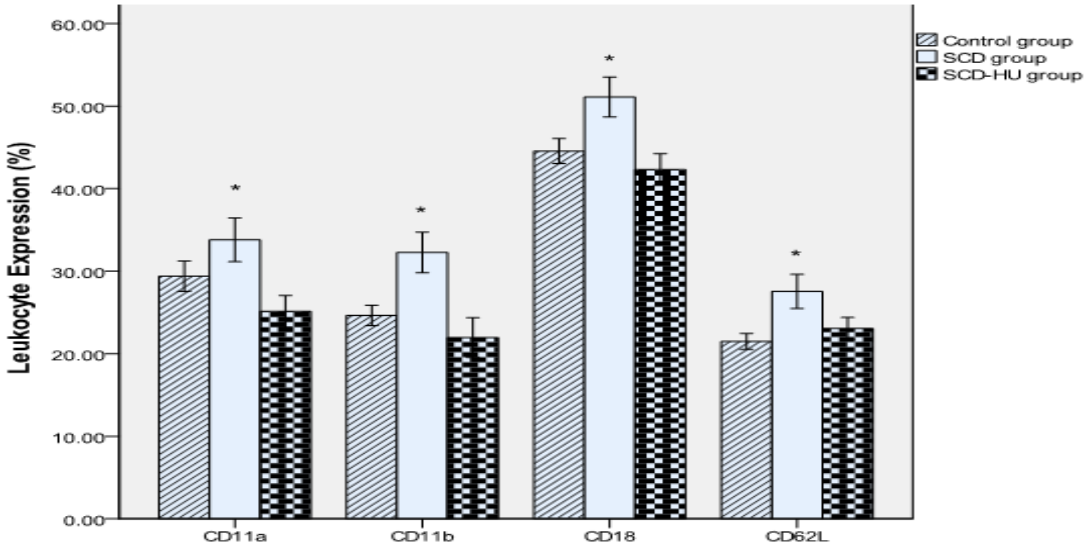

Figure 1: Leukocyte expression of different markers (Adhesion molecules) among Control group, sickle cell disease patients (SCD group) and sickle cell disease patients using hydroxyurea (SCD-HU group). Note: The expression of these markers increased in the patients but decreased after using hydroxyurea (*P*-value≤0.05)

DISCUSSION

WBCs play a core role in pathophysiology of SCD. Leukocytosis is a common phenomenon in this disease, and clinical evidence has shown that there is a direct correlation between leukocytosis and exacerbation of the disease, and leukocytosis as a risk factor increases the incidence of crises, such as acute chest syndrome, stroke, and early death [17]. In this study, there was a leukocytosis in patients. However, number of leukocytes returned to the normal level in patients after using HU. It seemed that the increase was mainly related to the neutrophils in patients, as the percentage of neutrophils in the peripheral blood decreased considerably after using HU. Other studies in this regard showed that HU decreased neutrophil count, and subsequently, leukocytosis decreased, and clinical signs were improved prior to a change in the level of hemoglobin F [18].

In this study, the expression of beta-2 integrin subunits, including CD11a, CD11b, CD18, and CD62L (L-selectin), on leukocytes was examined. The results revealed that all these markers increased in patients, and the use of HU could significantly decrease the expression of markers involved in the adhesion. Similarly, Hillary et al. concluded that adhesion molecules were reduced in patients receiving HU [19]. In this regard, Finnegan et al. in Boston found that the physical interaction between leukocytes and erythrocytes was effective in incidence of VOC, and the treatment with HU decreased the expression of adhesion molecules on WBC and reticulocytes and their interaction [20]. Therefore, treatment with HU significantly decreases the expression of adhesion molecules on leukocytes and also neutrophil count.

A study conducted by Okpala et al. to examine the correlation between worsening of the disease and expression of adhesion molecules on leukocytes showed that the expression of $\alpha M\beta 2$ (CD11b/CD18) on neutrophils and CD62L on neutrophils and lymphocytes significantly increased in patients with SCD. They also found that the expression of CD18 increased in patients who suffered SCD and nephropathy concurrently. The study also showed that the use of HU decreased the expression of integrins, such as $\alpha L\beta 2$ (CD11a/CD18) and CD11b on leukocytes. They concluded that the increased expression of $\alpha M\beta 2$ and CD62L exacerbated the disease. They also proposed that the improvement of clinical signs by HU before the increase in hemoglobin F was probably due to the reduction in expression of adhesion molecules by HU [10].

Contradictory results have been published about the expression of integrins on neutrophils in patients with SCD and showed that the expression of integrins in patients did not vary with that in normal people [22]. However, later studies revealed that the expression of CD11b on neutrophils and CD11a on lymphocytes was higher in patients with SCD than that in normal people [10]. All studies found that the expression of L-selectin

in initial stages of leukocyte adhesion to vessels was higher in patients who suffered SCD and VOC concurrently [10,21].

Leukocytes adhere to vascular wall through binding of integrin molecule subunits, such as CD11a, CD11b, CD18, and CD62L (L-selectin), to adhesion molecules of vessels happens through ICAM-1, Eselectin, P-selectin, and VCAM-1 [21,12]. The expression of these adhesion molecules on the endothelium of vessels probably happens after a vascular inflammation. Numerous inflammatory markers, such as TNFα, C-reactive protein, IL-8, and IL-1β, were reported to be in blood circulation of patients with SCD [23]. Pharmacological techniques that can reduce adhesion of leukocytes to the vascular wall may be an important strategy in prevention of VOC. In this regard, the reduction of serum E-selectin was reported in patients with SCD receiving HU [24]. Moreover, the adhesion of neutrophils to endothelial cells stimulated by TNFα in patients with SCD was reduced after using simvastatin [25]. The adhesion occurs through Mac-1, LFA-1, and VLA-4. It seems that HU can be effective in reduction of VOCs and mortality in patients with SCD through modulating adhesion molecules, decreasing leukocyte count, and reducing adhesion of RBCs to laminin through decreasing intracellular cAMP [26] as mentioned in recent reports.

Considering the increase in percentage of neutrophils in this study, it seems that neutrophils play an important role in pathogenesis of SCD, as the activity of these cells increase in the course of the disease besides their count. Lard et al. conducted a study on adhesion molecules on neutrophils in SCD in Holland and found that the expression of CD62L in patients decreased significantly compared to that in normal people, and the expression of CD64, which was an active neutrophil marker, increased in patients. Therefore, they proposed that neutrophils became active in those patients and were very effective in pathophysiology and incidence of crises [27]. The adhesion of active neutrophils to the wall of vascular endothelium cultured in the presence of TNFα highly increased. However, inhibition of leukocyte adhesion using an antibody against CD64 showed inconsistent results [28]. Other studies showed that the use of monoclonal antibodies against erythrocyte and leukocyte adhesion molecules inhibited their adhesion to the endothelium and decreased the risk of VOC in these patients [29]. In this respect, inhibition of adhesion molecules is effective in prevention of VOCs.

The present study concluded that leukocytes and adhesion molecules on them increased in patients with SCD. As leukocytosis and increased expression of adhesion molecules in patients with SCD decreased significantly after using HU, VOCs can be prevented by the use of HU as an important strategy in pharmacological approaches.

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