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Iron Chelation Reduces DNA Damage in Sickle Cell Anemia

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Abstract

Sickle cell anemia (SCA) is a blood condition that causes severe pain. One of the therapeutic agents used for the treatment of SCA is hydroxyurea, which reduces the episodes of pain but causes DNA damage to white blood cells. The aim of this study was to evaluate the efficacy of the combination of hydroxyurea and iron chelation therapy in relation to the extent of DNA-associated damage. Blood samples were collected from 120 subjects from five groups. Various hematological parameters of the obtained serum were analyzed. The amount of damage caused to their DNA was detected using the comet assay and fluorescent microscopy techniques. The percentage of DNA damage in the group that was subjected to the combination therapy (target group) was 1.32% ± 1.51%, which was significantly lower ($P < .05$) than that observed in the group treated with hydroxyurea alone (6.36% ± 2.36%). While the target group showed comparable levels of hemoglobin F and lactate dehydrogenase compared to the group that was treated with hydroxyurea alone, highly significant levels of transferrin receptors and ferritin were observed in the target group. The results of this study revealed that the administration of iron chelation drugs with hydroxyurea may help improve patients' health and prevent the DNA damage caused to white blood cells due to hydroxyurea. Further studies are needed to better understand the underlying mechanisms that are involved in this process.

Keywords

DNA damage, hydroxyurea, iron chelation therapy, leukocyte DNA, sickle cell anemia

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Introduction

Sickle cell anemia (SCA) results in leukocytosis accompanied with a chronic proinflammatory state. Mortality in SCA patients may be caused by severe recurrent infections and an elevation in the levels of inflammatory cytokines in their plasma. SCA is the most common type of hemoglobinopathy. The sickle cell trait occurs in heterozygous individuals who have one abnormal allele in their hemoglobin beta gene, while severe symptoms of sickle cell disease manifest in those who have two copies of an affected allele (homozygous).[1] It is the consequence of a point mutation caused by the replacement of adenine by thymine (GAG → GTG) in the sixth codon of the β-globin gene (βS) and the replacement of valine by glutamic acid in the polypeptide chain, which leads to the generation of an abnormal form of hemoglobin (HbS).[2] Several risk factors that have a statistically significant association with mortality have been

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identified among SCA patients, including low hemoglobin levels, liver variables (oxalacetic glutamic transaminase and alkaline phosphatase enzymes), and cardiovascular variables.[3]

Currently, two medications have been approved by the US Food and Drug Association (FDA) to counteract the associated painful vaso-occlusive episodes, one of which is hydroxyurea (HU). The use of HU decreases the hemoglobin polymerization that is caused in sickle cells due to the enhanced production of fetal hemoglobin and L-glutamine. New drugs as well as gene therapy are being evaluated in several clinical trials for their efficacy in the treatment of SCA, and positive results have been reported.[4,5]

The amino acid L-glutamine has also been prescribed and approved by the FDA for patients suffering from SCA. L-glutamine is essential for synthesizing nicotinamide adenine dinucleotide (NAD).[6,7] The levels of L-glutamine are affected by many factors, including stress and diseases, and, hence, supplementation is required. As an outcome of SCA, the uptake of L-glutamine is much higher in sickle red cells than in their counterpart normal red cells due to an increase in the intracellular NAD level. For this reason, the use of L-glutamine as a supplement for SCA patients is justified.[6,8]

HU is used for treating the complications associated with SCA and has been shown to improve the quality of the lives of SCA patients. The therapeutic effect that HU has is due to its ability to increase the production of fetal hemoglobin (HbF).[9,10] However, HU has several side effects, including the inhibition of both cellular proliferation and the activity of ribonucleotide reductase (RNR), which is an iron-dependent enzyme responsible for the synthesis of DNA. Several studies have reported that HU is genotoxic and potentiates DNA damage.[9] Iron chelation therapy is crucial for patients suffering from SCA, especially those who receive blood transfusion periodically to avert the liver damage caused by the high levels of ferritin.[11] Moreover, this therapy could rapidly reduce oxidative DNA damage.[12,13,14] The rationale behind this study was to investigate whether combining iron chelation therapy with HU reduces genotoxicity.

Materials and Methods

Study Sample

A comparative study was conducted with a total of 120 subjects (69 males and 51 females; 96 homozygous SCA patients and 24 non-diseased) who fulfilled the defined inclusion criteria. The inclusion criteria for this study dictated that the subjects be 10-40 years of age, present mild to moderate symptoms, have no active painful crisis, received folic acid, HU, or iron chelating agents for at least two years but not more than 10 years, and have undergone monthly blood transfusions. Patients who were less than 10 years old and more than 40 years old or who were diagnosed with acute painful attacks, acute chest syndrome, strokes, and complications of vascular occlusion a year before the blood sample collection were excluded from this study. In addition, those who had used any type of antibiotics for at least three months prior to the study, presented hemoglobin variants other than HbSS (such as HbSC and HbAS), or suffered from the human immunodeficiency virus (HIV) were also excluded from the study. Except for the non-diseased individuals, all the participants were patients who were being treated at the hematology clinic and day care unit at King Abdulaziz University Hospital and had been diagnosed with SCA. All subjects were divided into five groups ($n = 24$), as shown in Table 1. The first group (G1) consisted of non-diseased individuals, who were recruited from among blood bank donors, and the required samples were collected from them after their written consent had been obtained. The second group (G2) comprised SCA patients who had been treated with folic acid only, while the third group (G3) consisted of SCA patients who had been treated with HU. Further, the fourth group (G4) was made up of SCA patients who had been treated with an iron chelating agent only. Finally, the fifth group (G5) comprised SCA patients who had been treated with a combination of HU and iron chelating agents. The ethical approval for the study was obtained from the Ministry of Health of Saudi Arabia (Reference # H-02-J-002).

Table 1. Groups of the study.

Groups	Characteristics
G1	Healthy individuals with no SCA
G2	Individuals with SCA who were administered folic acid 5 mg/day.
G3	SCA patients who were subjected to HU at calculated dose 30 mg/kg/day.
G4	SCA with iron chelating agent only (Deferiprone 75-99 mg/kg/day- Deferoxamine 30-60 mg/kg over 8-24 h – Deferasirox 20-30 mg/kg/day).
G5	SCA with combination of hydroxyurea and iron chelation therapy

Evaluation of Genotoxicity of HU In Vitro

A series of different concentrations of HU (250, 500, and 1000 μM) were tested on two different blood samples and incubated for 18 h at 37°C. Immediately after the treatment period, the comet assay was conducted according to the recommended standard protocol assay.[15]

Blood Samples Collection

A 10-mL blood sample was collected from each subject in EDTA and plain tubes. The EDTA tubes were then used for the complete blood cell count (CBC) and the comet assay processes as described below. The blood samples in the plain tubes were left to clot and then centrifuged at 1500*g* for 10 min to collect the serum, which was stored at −80°C until used. The serum was utilized to analyze lactate dehydrogenase activity (LDH), human fetal hemoglobin (HbF), transferrin receptors (TfR), and ferritin. The levels of the HbF and TfR were determined using commercial ELISA kits (Elabscience, Housten, TX, and Abcam, Cambridge, UK).

Single-Cell Gel Electrophoresis (Comet Assay) and Comet Scoring

The comet assay, also known as the single-cell gel electrophoresis assay, is an easy and sensitive technique that enables a quantitative assessment of the effects of DNA damage or apoptosis induction and the underlying mechanisms involved in different target cell types. The assay has also been utilized in studies regarding DNA repair.

The comet assay is based on cell migration through electrophoresis and the quantitative analysis of populations of targeted cells. In general, cells are suspended in an agarose gel that is layered onto a microscope slide. Electrophoresis causes the migration of unwound or fragmented DNA out of the cell's nucleus, which results in a characteristic, comet-like shape and appearance that can be easily seen microscopically with fluorescent staining of the DNA. The measurement and quantitative analysis of the fluorescent intensities revealed by the cells gives masseurs of cellular DNA content distribution and shows damage if present. The single-cell gel electrophoresis (comet assay) was carried out to characterize and quantify the degree of DNA damage under an alkaline condition as described by others but with slight modifications.[16] Approximately 1-2 $\times$ 10^4 leukocytes were combined with 100 µL of melted agarose (LMA), and 75 µL of this solution was immediately pipetted onto the comet slide (Trevigen, USA). Next, the slides were immersed in a freshly prepared cold lysing solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris, pH 10, 1% sodium sarcosinate, 1% Triton X-100, and 10% DMSO) for a minimum of 1 h at 4°C. The slides were then placed in a horizontal electrophoresis apparatus filled with freshly prepared alkaline solution (pH $\geq$ 13). The slides were immersed in the lysing solution for 20 min before electrophoreses was performed at 1 volt/cm for 20 min. Next, the slides were rinsed three times for 5 min in the washing buffer (0.4 M of Tris at pH 7.4) and immersed in absolute ethanol for 5 min. After air drying, the slides were stained with ethidium bromide (0.5 µg/mL), covered, and analyzed.

Each sample was processed in duplicate to avoid processing errors. The slides were then examined under a fluorescence microscope for comet scoring. The extent of the DNA damage was measured quantitatively using a comet analysis system (Loats, USA) based on extended dynamic range imaging (EDRI) technology, which expressed the comet tail moment automatically. Parallel to each experiment, a series of negative controls were carried out to determine any non-specific formation or reduction in the comet. The percentage of the comet cells were calculated as follows:

$$\frac{\text{Comet cells count}}{\text{Total cells counts}} \times 100$$

The means and standard deviations (SDs) for the tail moment values for all images in each group that was generated using EDRI technology were calculated.

Statistical Analysis

All the numerical results shown have been illustrated as mean $\pm$ SD. A one-way variance analysis (ANOVA) has been used along with an independent sample *t*-test to identify the difference between the groups using SPSS version 23.

Results

Evaluation of Genotoxicity of HU In Vitro

Figure 1 illustrates the results of the comet assay with HU. After treatment for 18 h, it was clear that the count of the damaged cells had increased for all concentrations tested and in a dose-dependent manner as compared to the control.

Hematological Parameters

A summary of the tested hematological parameters for all experimental groups has been presented in Table 2. All experimental groups showed significantly elevated levels of white blood cells (WBCs) (*P*-value <0.05) compared to the non-diseased group (G1), although G5 showed slightly higher levels than G2 and G3 groups. With regard to red blood cell (RBC) count, the patients who were subjected to HU and/or chelating agents presented the lowest value among all the groups. Furthermore, all treated groups, when compared to the G1, had significantly reduced RBC counts (*P*-value <0.05). All the SCA groups had comparable levels of Hb that were significantly lower as compared to G1.

As statistically tested, the differential WBCs expressed significant elevated levels of neutrophils in the G5 group relative to all groups, including G1. The eosinophils levels in G5 were comparable to all groups except G4, which had the greatest amount out of all the groups. None of the groups showed any significant differences in basophils, with a slight rise in G2. The volume of lymphocytes and monocytes was the greatest in G5 and was not significantly different for G3 and G4.

Serum Analysis

Table 3 shows the levels of the serum analysis parameters of the experimental groups. The HbF levels for both G3 and G5 were significantly greater than those of all other groups, while they were the lowest for the G2 group. In fact, the HbF amount was increased in the groups treated with HU and was significantly higher compared to the G4 group. In addition, the SCA groups (G2, G3, G4, and G5) presented LDH activity that was considerably greater than the normal group, while the activity of LDH was only slightly raised in the G4 and G5 groups when compared to G2. Furthermore, the transferrin receptors in the G4 and G5 groups were significantly more in relation to all other groups. Moreover, when compared to the G1 and G2 groups, the ferritin levels were found to be significantly increased in the G4 and G5 groups and slightly elevated in G4 as compared to the G3 group.

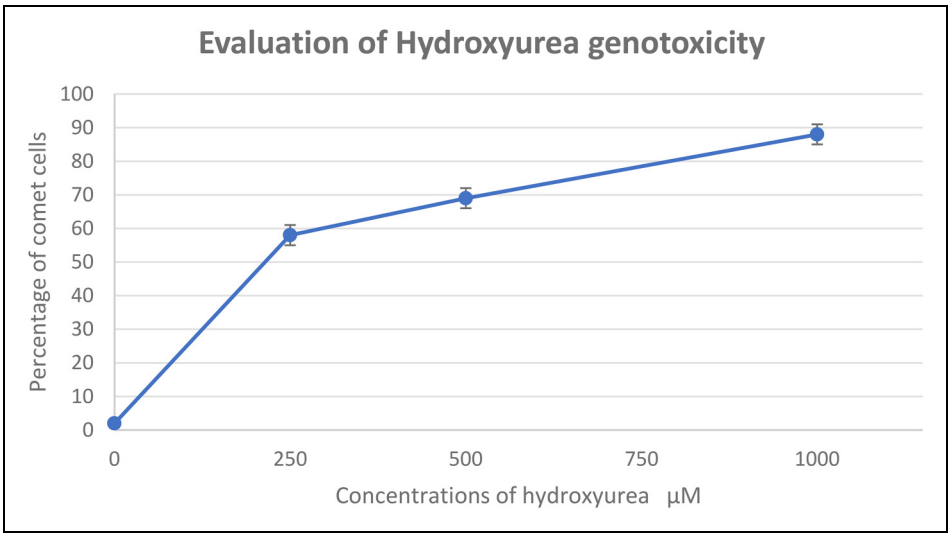

Figure 1. Evaluation of the effects of different concentrations of hydroxyurea on genotoxicity.

Comet Assay and Comet Scoring

The genotoxicity of the drugs involved was assessed instead the comet assay (expressed cells with comet percentage and tail moment are shown in Table 4). About 300 leukocytes were analyzed for comet formation and counted using fluorescent microscopy. As statistically tested, the count of the normal leukocytes in G5 were comparable to that of G1. However, G5 and G4 have only a few discrepancies relative to G3. In terms of the percentage of the comets, the patients treated with HU present the highest comet percentages of all the treated groups, with an average tail moment of 1.94 ± 0.3. The G5 group presented higher values for comet percentage, by about 75%, when compared to the G3 group, which had an average tail moment of 0.456 ± 0.35. The lowest comet percentages were observed in the G1 and G4 groups. The images, which were generated using EDRI technology, are shown in Figure 2.

Discussion

Sickle cell disease (SCD) produces recurrent, severe episodes of pain and extensive organ damage, caused by the occlusion of microcirculation.[17] Some patients may develop severe complications, such as pulmonary hypertension, priapism, stroke, leg ulceration, acute chest syndrome, bone necrosis, and severe hemolytic anemia, which makes it an important topic for research. In SCD, RBCs with abnormal hemoglobin aggregates deform to take on a characteristic sickle shape and become significantly less elastic, which causes them to get stuck inside the capillaries, thus obstructing blood flow and causing hypoxic ischemic organ damage.[18] The amount of fetal hemoglobin (percentage of HbF) is the most crucial factor. HbF is protective against clinical severity; a low percentage of HbF is associated with a higher risk of experiencing vaso-occlusive complications, organ damage, and early death. Accordingly, the pharmacologic induction of HbF is a logical treatment objective for patients suffering from SCA, and recent studies have focused on this topic.[19]

HU is the most used drug for the treatment of SCD, while iron chelators are mainly used for the treatment of those SCD patients who frequently undergo blood transfusions. For this reason, considerably less data is available on the use of iron chelators for the treatment of SCA. The role of iron chelators is to reduce the liver damage caused by the presence of iron in the blood, which is commonly seen in SCA patients.[20] This is because chelation therapy with deferoxamine is effective for preventing the risk of transfusion iron overload. Moreover, iron chelation therapy could prevent oxidative DNA damage.[21]

Previously published studies have focused on the use of HU for the treatment of SCA only and its effects on DNA damage in SCA patients. Thus far, only a few studies have investigated the effect of HU on the response of the various hematological parameters in the serum. It should be noted that no published data were found regarding the administration of a combination therapy that uses HU with iron chelators or regarding their effects on DNA damage.

A study conducted in Iraq on SCA patients indicated that significant differences were noted between the hematological parameters of the participants in the group treated with HU and those of the control group. For instance, the average amount of hematocrit in the HU group was significantly higher than that in the control group. In addition, there was a significant decline in WBCs and platelet count in the HU group as compared to the control group.[10,22] Protein C and free Protein S are reduced during the crisis, with a lower number of platelet microparticles and a higher percentage of markers of endothelial damage and of red cell origin. During a chest crisis, adrenomedullin and endothelin-1 (ET-1) were elevated, suggesting that the therapy inhibited ET-1inchestcrisis. Such a decrease may be associated with endothelial damage.[10] The lower platelet number may be due to the aggregation of many cells to the surface, which was coated with

Table 2. The effect of the drugs on hematological parameters.

Groups	G1	G2	G3	G4	G5
WBCs (10^3/μL)	6.43 ± 0.75	9.12 ± 2.33^{c}* *P*-value (0.0017)	10.35 ± 3.48^{a}* *P*-value (0.03)	12.2 ± 2.56	12.67 ± 3.18
RBCs (10^6/μL)	4.97 ± 0.53	3.77 ± 0.93^{c}* *P*-value (0.0002)	3.40 ± 0.63^{a}* *P*-value (0.009)	2.87 ± 0.82	2.59 ± 0.38
Hb (g/dL)	13.20 ± 1.74	9.0 ± 0.4	8.55 ± 0.91	7.74 ± 1.11	8.01 ± 0.38
Neutrophils (10^3/μL)	2.75 ± 0.85	3.95 ± 0.5^{c}* *P*-value (0.0000)	5.2 ± 0.35^{a}* *P*-value (0.0000)	5.7 ± 1.7^{b}* *P*-value (0.0001)	8.18 ± 1.96
Eosinophils (10^3/μL)	0.24 ± 0.15	0.29 ± 0.17	0.25 ± 0.16	0.62 ± 0.49^{b}* *P*-value (0.018)	0.33 ± 0.13
Basophils (10^3/μL)	0.05 ± 0.02	0.11 ± 0.09	0.08 ± 0.03	0.06 ± 0.02	0.07 ± 0.02
Lymphocytes (10^3/μL)	2.75 ± 0.74	2.93 ± 1.28^{c}* *P*-value (0.022)	3.47 ± 2.17	3.95 ± 1.29	4.18 ± 0.87
Monocytes (10^3/μL)	0.56 ± 0.14	1.09 ± 0.72	0.95 ± 0.45	1.19 ± 0.56	1.33 ± 0.82

[a]There is a significant difference between G5 and G3 groups.
[b]There is a significant difference between G5 and G4 groups.
[c]There is a significant difference between the G5 and G2 groups.

Table 3. The effect of drugs on serum analysis parameters.

Groups	HbF(ng/mL)	LDH(U/L)	Transferrin receptors (pg/ml)	Ferritin(ng/ml)
G1	Non detect	158.54 ± 16.51	450.16 ± 255.77	106.20 ± 59.3
G2	33 ± 5^{c}**	430.62 ± 50.44^{c}*	736.98 ± 564.43^{c}***	259 ± 179.94^{c}***
G3	437.28 ± 12.2	466.92 ± 40.17	1264.67 ± 857.58^{a}***	613.61 ± 237.55^{a}***
G4	75 ± 9.1	584.23 ± 14.72	5391.8 ± 2541.88	5199.57 ± 828.37^{b}*
G5	372.46 ± 5.65	557.08 ± 77.31	5199.57 ± 1844.95	2484.42 ± 1373.76

Data were expressed as $\bar{X} \pm$ SD.
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[a]There is a significant difference between G5 and G3 groups.
[b]There is a significant difference between G5 and G4 groups.
[c]There is a significant difference between the G5 and G2 groups. *$0.01 \leq P$-value <0.05, **$0.001 \leq P$-value <0.01, ***P-value <0.0001.

fibrinogen, von Willebrand factor (vWF), and collagen, thus supporting the idea of platelet–endothelial adhesion.[23]

The effects of HU on the hematological parameters revealed that it could result in higher levels of hemoglobin, hematocrit, and mean corpuscular volume (MCV) and a decline in leukocyte and neutrophil numbers.[24] Further, a significant increase was observed in the activity of the LDH enzyme in the SCA group as compared to the control group. All the HU-treated groups demonstrated a significant decline in LDH levels in comparison to the SCA group.[24] It was noted that SCA patients had significantly higher leukocyte and neutrophil counts and that HU resulted in not only an increased concentration of HbF but also a decreased number of these cells.[24]

The results of another study that compared the treatment with either HU or chronic transfusion therapy indicated that the HU group presented a significantly higher HbF percentage than the other group.[25] In a large study, which was conducted for more than two years and included 33 centers in Canada and the USA, it was demonstrated that the serum ferritin levels were lower when treatment was commenced using two iron chelators: deferasirox and deferoxamine.[26]

An increased WBC count has also been associated with poor clinical treatment outcomes, while elevated LDH levels, which reflects intravascular hemolysis, have been associated with morbidity and mortality in the context of SCA. The results of this study indicated that HU caused severe damage to WBCs, which is consistent with the findings of Flanagan et al.[27] The severity of the damage is associated with HU's ability to inhibit RNR, which leads to the reduction of DNA biosynthesis, and the inactivation causes DNA replication stress and DNA damage.[28] Regarding the serum LDH changes, the LDH levels were significantly high for all SCA patients in the different groups. LDH is a soluble enzyme located in the cytosol of the cells and tissues. It is abundant in RBCs and can reflect the hemolysis or cell damage when released in the surrounding environment. Therefore, LDH levels rose in our SCA patients due to the lysis of erythrocytes, and this is strongly correlated with the reduced counts of RBCs as revealed by this study.[29]

Table 4. The effect of the drugs on leukocytes, which was counted by fluorescent microscope. Data were expressed as $\bar{X} \pm SD$.

Groups	Normal cells	comet cells	Percentage of comet %
G1	246.8 ± 35.80	2.1 ± 1.66	0.85 ± 0.63
G2	236.8 ± 52.65	2.5 ± 2.07	1.12 ± 1.05
G3	278.2 ± 49.68^{a}**	18.5 ± 9.42^{a}***	6.36 ± 2.36
G4	227.7 ± 52.19	2.5 ± 1.78	1.04 ± 0.78
G5	213 ± 60.92	3.2 ± 0.55	1.32 ± 1.51

[a]There is a significant difference between G5 and G3 groups.
[b]There is a significant difference between G5 and G4 groups.
[c]There is a significant difference between the G5 and G2 groups. *$0.01 \leq P$-value <0.05, **$0.001 \leq P$-value <0.01, ***P-value <0.0001.

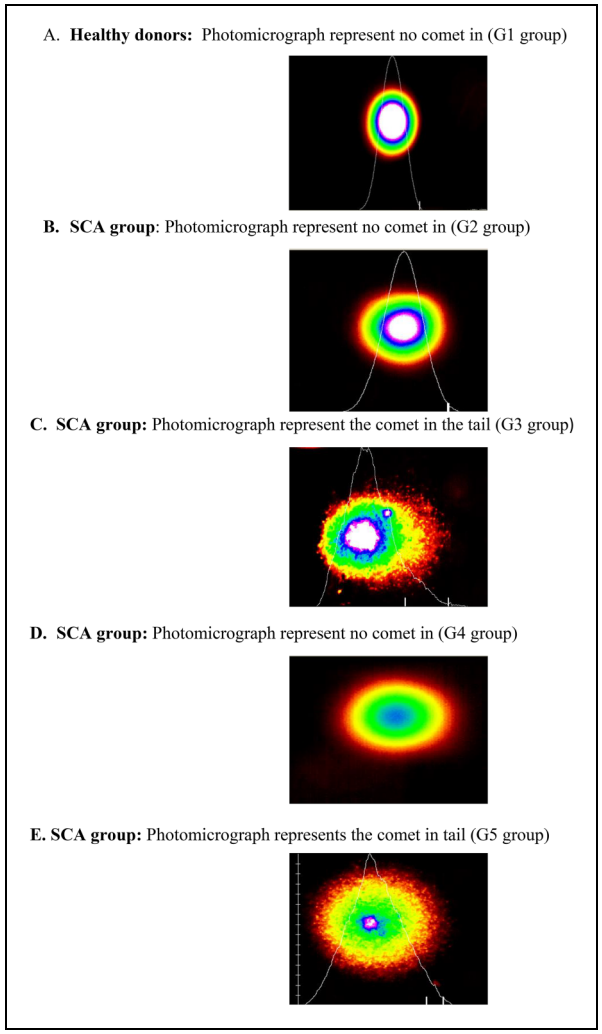

Figure 2. Photomicrographs which generated by EDRI technology for the comet damage.

As mentioned in previous studies, the therapeutic effect of HU can increase the production of HbF.[30] In our study, HbF was extremely high in the serum of the patients from the HU group as compared to those from the other groups, which is in accordance with the results of the study conducted by Nkya et al.[31] The group treated with HU had the highest level of HbF, followed by the G5 group (HU and chelating agent). The HbF has the capability of replacing the mutated β-globin with a γ-globin chain, which is non pathological, hence decreasing the number of vaso-occlusive events and infarctions.[32] It has been reported that the administration of HU causes an elevation in the levels of Protein C and S, which may be associated with a downregulation of the thrombotic effects cascade.[10]

Several studies have found evidence to support the contribution of leukocytes to the disease process in sickle hemoglobinopathies.[33] The correlation between the increased levels of WBCs and SCD have been proved on a biological basis. The therapeutic implications of this relationship have conferred clinical benefits for SCD treatment. Our study revealed that the levels of WBCs were elevated in SCA patients in all groups as compared to those from the normal group, which corresponded with the severity of the disease.

Interestingly, the neutrophil counts also increased in patients who were administrated HU and/or chelating agents. This is in contradiction with what has been reported by Kono et al[23] that DFS treatment decreased the number of neutrophils with NET formations.[23] The highest counts of neutrophils were observed in the HU group, which indicates a good response and efficacy of the combination therapy according to Charache and Wang.[34] This research also illustrated that the level of DNA damage for SCA patients treated with HU only was significantly higher than that for those treated with a combination of HU and chelating agents. For this reason, the combination of both HU and iron chelation agents should be considered in future clinical trials and the underlying mechanism should be elucidated further. In this study, we investigated whether there was a significant difference between the HU-treated group and the other combination group of HU with iron chelation in terms of cytotoxicity. Despite no baseline value being recorded before the treatment started, which is a limitation of this study, the results indicated a significant reduction in the combination group versus the HU group. This may indicate that such a combination could be beneficial for the future treatment of SCA patients.

Conclusions

In the light of available evidence, the combination therapy of HU and iron chelation ameliorated the DNA damage caused to leukocytes, as estimated by the comet assay and the elevated number of neutrophils. Therefore, more clinical studies that use more SCA patients are needed, which may provide more conclusive results regarding the potential effect of the combination of HU and chelation therapy for SCA patients.

Limitations of the Study

One of the limitations of this study is that there was no baseline parameter recorded, and this was retrospectively cross-sectionally done. It was also extremely difficult to recruit patients for this study. This was carried out by including

those who have satisfied the inclusion and exclusion criteria. Based on the results, there is no conclusive evidence that the combination therapy alone resulted in the enhancement effect, and other factors may also be involved.

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Informed Consent

Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

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