Cytotoxicity and DNA damage in the neutrophils of patients with sickle cell anaemia treated with hydroxyurea

Alano Martins Pedrosa¹*, Maritza Cavalcante Barbosa¹, Thayna Nogueira dos Santos¹, Luzia Kalyne Almeida Moreira Leal¹, Amanda de Araújo Lopes¹, Darcielle Bruna Dias Elias¹, Greyce Luri Sasahara¹, Bruno Coêlho Cavalcanti², Romélia Pinheiro Gonçalves¹

¹Pharmacy Department, Faculty of Pharmacy, Odontology and Nursing, Federal University of Ceará, Fortaleza, CE, Brazil, ²Physiology and Pharmacology Department, Faculty of Medicine, Federal University of Ceará, Fortaleza, CE, Brazil

Hydroxyurea (HU) is the most important advance in the treatment of sickle cell anaemia (SCA) for preventing complications and improving quality of life for patients. However, some aspects of treatment with HU remain unclear, including their effect on and potential toxicity to other blood cells such as neutrophils. This study used the measurement of Lactate Dehydrogenase (LDH) and Methyl ThiazolTetrazolium (MTT) and the comet assay to investigate the cytotoxicity and damage index (DI) of the DNA in the neutrophils of patients with SCA using HU. In the LDH and MTT assays, a cytoprotective effect was observed in the group of patients treated, as well as an absence of toxicity. When compared to patients without the treatment, the SS group (n=20, 13 women and 07 men, aged 18-69 years), and the group of healthy individuals (AA) used as a control group (n=52, 28 women and 24 men, aged 19-60 years), the SSHU group (n=21, 11 women and 10 men, aged 19-63 years) showed a significant reduction (p<0.001) in LDH activity and an increase in the percentage of viable cells by the MTT (p<0.001). However, the SSHU group presented significantly higher DI values (49.57±6.0 U/A) when compared to the AA group (7.43 ± 0.94U/A) and the SS group (22.73 ±5.58 U/A) (p<0.0001), especially when treated for longer periods (>20 months), demonstrating that despite the cytoprotective effects in terms of cell viability, the use of HU can induce DNA damage in neutrophils.

*Correspondence: A. M. Pedrosa. Laboratório de Hematologia, Faculdade de Farmácia, Universidade Federal do Ceará. Rua Capitão Francisco Pedro, 1210 - Rodolfo Teófilo, 60370-430 - Fortaleza – CE, Brasil. E-mail: alano_martins@yahoo.com.br
INTRODUCTION

Sickle cell anaemia (SCA) is a hereditary disease caused by the substitution of glutamic acid with valine in the sixth codon of the $\beta$-globin leading to the formation of haemoglobin S (HbS). The sickle red blood cell is rigid and inflexible, favoring the occurrence of vaso-occlusive and haemolytic events and starting a chain reaction that culminates with the generation of reactive oxygen species (ROS), reduced bioavailability of nitric oxide (NO) and the chronic inflammatory process, with direct involvement of neutrophils in the early stages and propagation of these mechanisms (Okpala, 2004; Conran et al., 2007; Rees, Gibson, 2011; Chirico, Pialoux, 2012).

The leukocytes play an important role in the pathophysiology of SCA. The vaso-occlusive process and endothelial injury result in a chronic inflammatory response characterized by high levels of proinflammatory cytokines, which are capable of making the activated endothelium. The endothelial activation results in increased expression of adhesion molecules on neutrophils, such as E-selectin, P-selectin and ICAM-1 (Intercellular Adhesion Molecule-1), inducing the chemotaxis of neutrophils and its interaction with adhesion molecules on sickle red blood cells, other leukocytes and platelets, leading to pancellular activation resulting in the release of most proinflammatory cytokines. Thus, there is a vicious circle between production of inflammatory mediators and cellular adhesion to the endothelium, leading to a state of chronic inflammation, fundamental to the process of vaso-occlusion (Okpala, 2006; Canalli et al., 2008; Conran, Franco-Penteado, Costa, 2009; Segel, Halterman, Lichtman, 2011).

The high count of neutrophils, often seen in patients with SCA, even in the absence of infection, has been associated with the occurrence of painful crises, acute chest syndrome (ACS), cerebrovascular accident (CVA) and early death; however, the mechanisms responsible for this are not completely understood (Platt et al., 1994; Castro et al., 1994; Ohene-Frempong et al., 1998). Some factors that may cause the interruption of apoptosis, such as proinflammatory cytokines, cell adhesion, hypoxia, and transmigration of bacterial lipopolysaccharide (LPS) may be related to the increased number of neutrophils and the survival time of these cells, suggesting an important relevance to the pathogenesis of SCA, since changes in the processes of apoptosis may affect cellular function and increase the damage potential (Ohene-Frempong et al., 1998; Moulding et al., 1998; Cross et al., 2005; Cross et al., 2006; Conran et al., 2007).

Hydroxyurea (HU) is the only drug approved by the U.S. Food and Drug Administration (1999) and European Medicines Agency (2008) for the treatment of SCA. HU inhibits the enzyme ribonucleotide reductase (RNR), causes cell-cycle arrest, and allows globin genes to be more actively expressed. By killing cycling cells, HU changes the kinetics of erythroid proliferation, forcing more F cells to be produced from primitive progenitors and directly stimulating HbF production (Franco et al., 2006). Furthermore, HU therapy increases haemoglobin concentration, reduces the expression of adhesion molecules on erythrocytes, platelets and neutrophils, decreases the production of granulocytes and contributes to the improvement of clinical events, reducing the number of hospital admissions, the frequency of painful episodes, the need for transfusion and the occurrence of CVA and ACS (Platt et al., 1984; Steinberg et al., 2003; Zago, Pinto, 2007; Cartron, Elion, 2008; Orah, Platt, 2008; Conran, Franco-Penteado, Costa, 2009; Lou et al., 2009).

Despite these benefits the HU therapy may have clastogenic, mutagenic and teratogenic effects (Murphy, Chaube, 1964; Oppenheim, Fishbein, 1965; Aliverti, Bonanomi, Giavini, 1980; Ware, Aygun, 2009). In the literature, the HU ability to cause cancer is controversial and the long-term efficacy and safety of HU in treating patients with SCA remains inconclusive (Steinberg et al., 2010). Some studies have shown that HU is genotoxic while other studies suggest that HU has low mutagenicity in vivo (Hanfl et al., 2000; Friedrisch et al., 2008). Furthermore, some reports relate that HU acts as a competitive inhibitor of catalase-mediated hydrogen peroxide decomposition and this effect could be related to in vivo toxicity (Juul et al., 2010).

The long term safety of using HU remains an important issue, especially regarding genotoxicity and cell damage, and impact on the function of different organs (spleen kidneys, brain, lings) (Cançado et al., 2009).

Thus, some aspects regarding the treatment with HU and the safety of its long term use are still not fully elucidated, especially its effect on other blood cells other than erythrocytes. Therefore, it is necessary to evaluate the effect of HU regarding the genotoxicity and cytotoxicity in neutrophils isolated from patients with sickle cell anaemia at baseline, seeking evidence about the effect of HU on these cells and its influence on the modulation of the chronic inflammatory process in these patients.

MATERIAL AND METHODS

Criteria for inclusion

Subjects and samples

This is a cross-sectional study of 41 adult patients
(24 women and 17 men, aged 18-69 years) with clinical and laboratory diagnosis (confirmed by haemoglobin electrophoresis and molecular biology) of sickle cell anaemia (homozygotic SS form), representing 50% of the patients attending the outpatient unit of the haematology service of a reference hospital in Fortaleza, CE, Brazil (Table I presents the characteristics and clinical details of all individuals participating in the study). The study included SCA patients in a stable condition, according to the criteria of Ballas: absence of painful episodes and/or intercurrent illnesses, such as infections and inflammations in the four weeks preceding the study; no hospital admissions in the 2-3 days preceding the study and no blood transfusions in the four months preceding the study (Ballas, 2012).

In Brazil, the Ministry of Health degree nº 872 of 6th November 2002 approved the use of HU for patients with sickle cell disease. The drug is recommended for patients with a laboratory diagnosis of sickle cell disease (HbSS, S/β Thalassemia, HbSC, Hb SD), including children with three or more episodes of vaso-occlusive crises requiring hospital medical care; a recurring acute chest crises; one or more strokes; recurrent priapism and severe and persistent anaemia in the past 12 months, Hb concentration consistently less than 7 g/dL, concentration of fetal Hb<8% after 2 years of age, WBC count 20x10^9/L in the absence of infection. Patients with hypersensitivity to HU will be excluded from this protocol, as well as patients with presence of at least one of the following items related to bone marrow dysfunction: neutrophils <2.5x10^9/L, platelets <95x10^9/L, Hb concentration <4.5 g/dL or reticulocyte count <95 x 10^9/L, pregnant women and HIV-infected patients (Brazil, 2002; Cançado et al., 2009).

The selected patients were divided into two groups according to the use of HU: SS (without HU treatment) (n=20, 13 women and 07 men, aged 18-69 years) and SSHU (receiving oral HU dose between 15 and 25mg/kg/day) (n=21, 11 women and 10 men, aged 19-63 years). A control group (AA) was made up of 52 healthy blood donors (28 women and 24 men, aged 19-60 years) from a blood center in Fortaleza, CE, Brazil. Exclusion criteria for all groups were: presence of infectious diseases (HIV-1 and 2, HBsAg, HCV and HTLV-1 & 2), pregnancy, presence of renal failure or liver disease, smoking, alcohol consumption, use of chelated iron, anti-inflammatory drugs, antioxidant vitamins, or any immunosuppressant. The survey was approved by the Ethics Committee of the Federal University of Ceará (COMEP/Protocol number 101/12), and all subjects signed an informed consent form. Samples (10 mL) of peripheral blood were collected with EDTA for molecular biology. Samples (10 mL) of heparinized peripheral blood were collected for isolation of polymorphonuclear leukocytes, predominantly neutrophils.

### Molecular biological analysis

Molecular diagnosis of patients was based on a Polymerase Chain Reaction for mediated Restriction Fragment Length Polymorphism (PCR-RFLP), by digestion with DdeI restriction enzyme, according to the methods of Saiki et al. (1985).

### Isolation of polymorphonuclear leukocytes

The isolation of polymorphonuclear leukocytes was carried from whole blood by differential gradient

| Table I - Characteristics and clinical details of the control group and patients with sickle cell anemia participating in the study |
|---------------------------------|----------------|----------------|
| **Male/Female**                 | **AA (n= 52)** | **SS (n= 20)** |
| Age (years)                     | 24.6 (19; 60)  | 30 (18; 69)    |
| Red blood cell count (x10^{12} /L) | 4.6 (4.2; 5.7) | 2.66 (1.77; 4.0) |
| Haemoglobin(g/dL)               | 13.5 (12.2; 15.2) | 8.4 (5.8; 11.5) |
| Haematocrit(%)                  | 41 (37; 49)    | 24.33 (16.9; 32.9) |
| MCV (fL)                        | 87 (79; 92.1)  | 91.1 (74.4; 107.6) |
| MCH (pg)                        | 28.9 (25.9; 31.6) | 31.5 (28.7; 37.7) |
| White blood cell (x10^{9} /L)   | 5.7 (4.1; 8.4)  | 13.7 (5.5; 22.1) |
| Neutrophil(%)                   | 47 (39; 51)    | 59 (39; 85)    |
| Reticulocytes(x10^{3} / µL)     | UD             | 272.5 (110.9; 597.8) |
| Platelets(x 10^{9} /µL)         | 220 (148; 312) | 371 (154; 798) |
| HbF(%)                          | UD             | 5.66 (1.0; 13.8) |

AA: Control group (healthy individuals); SS: patients with sickle cell anaemia not treated with HU; SSHU: patients with sickle cell anaemia treated with HU; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Haemoglobin; UD: Undetermined. The figures presented (except M/F) represent the average (minimum and maximum).
using a gelatin solution, following the methodology proposed by Henson (1971) and modified by Lucisano and Mantovani (1984) in which the cells were suspended in buffered Hank’s balanced solution, containing calcium and magnesium. The preparations contained predominantly neutrophils (85.0 ± 2.8%).

Cell viability assay

Cell viability was determined by a Trypan Blue dye exclusion assay, in which the neutrophil suspension (2.5x10^6/mL) was mixed with an equal volume of Trypan Blue dye, 0.1%, and transferred to a Neubauer chamber. The Trypan Blue dye incorporated only non-viable cells due to membrane lesions (Lucisano, Mantovani, 1984). The proportion of viable cells was estimated by counting 200 cells in an optical microscope.

Cytotoxicity assays

The cytotoxicity was analyzed by lactate dehydrogenase (LDH) release, which assesses cell death by necrosis, and by the MethylThiazolTetrazolium (MTT) assay, measuring cellular metabolic activity through the pathway succinate-tetrazolium redutase, enzyme active only in cells with intact respiratory metabolism, which reduces the tetrazolium salt (yellow coloration) to a salt purple colour (formazan) (Renzi, Valtolina, Forster, 1993).

After isolation, a suspension of cells (2.5 × 10^6/mL) was incubated with buffered Hank’s balanced solution (test group) or 0.2% Triton X-100 (known to cause cell lysis and used as a positive control), for 30 min at 37°C. The activity of LDH was determined in the supernatant according to the manufacturer’s instructions (LDH liquidum of Labtest Diagnosis, MG, Brazil) in which the consumption of NADH was monitored by conversion of pyruvate to lactate at 340 nm. A solution of MTT at a concentration of 10 mg/mL was added to a cell suspension of neutrophils (5.0x10^6/mL) for the colorimetric determination of the formazan crystals at 540 nm, according to the protocol described by Mosmann (1983).

Comet assay

The standard alkaline protocol for comet assay was used, as reported by Singh et al. (1988). The assay was performed in accordance with general guidelines for in vivo use of the comet assay (Tice et al., 2000; Hartmann et al., 2003). Briefly, the neutrophil suspension (2.5x10^6 cells/mL) was mixed with low-melting-point agarose and spread onto glass slides pre-coated with agarose, and cover slips were gently placed over their content. Once the samples solidified, the cover slips were removed and the slides were soaked in freshly made, chilled lysis solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.2, to which 1% Triton X-100 and 10% DMSO had been added) for 1-2 days under refrigeration. Excess liquid was blotted away from each slide’s back and edges; the slides were then transferred to an electrophoresis tank, and an alkaline solution (300 mM NaOH, 1 mM EDTA, pH>13) was added. The slides were exposed to the alkaline solution for 20 min to allow for DNA unwinding and for the expression of alkali-labile sites as single strand breaks. DNA was then electrophoresed for 20 min (25 V; 300 mA; 0.9 V/cm). Slides were removed from the electrophoresis tank, cleaned, washed three times (5 min each time) with neutralization buffer (0.4M Tris, pH 7.5), washed three times with distilled water, and allowed to air dry. All steps of the assay were conducted under dim light.

Slides were then fixed and silver-stained according to the methods of Nadin, Vargas-Roig and Ciocca (2001). For evaluation of DNA damage, 100 cells per subject were analyzed at 200x magnification under a light microscope. Cells were assessed visually and received scores from 0 (no migration) to 4 (maximum migration) according to tail intensity (size and shape). Therefore, the total scores (damage index or DI) for 100 cells ranged from 0 (all cells with no migration) to 400 (all cells with maximum migration). Slides from patients and controls were processed, coded, mixed, and evaluated together.

Statistical analyses

The Statistical Package for the Social Sciences (SPSS) 10.0 for Windows was used for all data analyses, and graphs were made with the GraphPad Prism 4.0 for Windows. The level of significance was 0.05 for all tests, and the data is presented as mean ± S.E. of the mean (SEM). The Kolmogorov-Smirnov test was used to check for normal distribution of the data. The Analysis of variance (ANOVA) followed by the Tukey post-test was used to determine statistical differences between groups AA, SS and SSHU. The parametric T-test was used to test the influence of the mean dose of HU and HU Length of treatment on LDH release, MTT reduction and DI values. The mean dose of HU and the treatment length were separated into two categories according to their medians.

RESULTS

Influence of treatment with HU on cell viability evaluated by exclusion assay with Trypan Blue dye in neutrophils from patients with sickle cell anemia

Table II shows that the number of viable cells of the SS group (93.7±0.34%) was significantly lower than
in the AA group (95.8±0.29%) (p<0.05). The number of viable cells in the SSHU group (95.0±0.42%) was similar to the SS and control groups, i.e., there was no significant difference.

**Influence of treatment with HU on LDH release and MTT reduction in neutrophils from patients with sickle cell anaemia**

Table III shows that the SS group (10.25 ± 1.21 U/L) showed a greater LDH release compared to the AA group (6.71±0.68 U/L) and the SSHU group (5.59±0.37 U/L) (p=0.0003). The LDH release did not differ between the SSHU and the AA groups (p>0.05). In the experimental model MTT reduction, the SS group (35.14±2.32%) showed a lower number of viable cells when compared to the AA group (56.8±3.2%) and the SSHU group (50.59 ± 3.64%) (p<0.0001). There was no significant difference between the number of viable cells in the SSHU and the AA groups (p>0.05). The mean dose of HU and the Length of HU treatment did not affect the LDH release and MTT reduction (Table IV).

**Influence of treatment with HU in the DI values in neutrophils from patients with sickle cell anaemia**

Figure 1 shows an association between DI values and treatment with HU. DI values for the SSHU group were greater (49.57 ± 6.0 U/A) when compared to the AA group (7.43± 0.94 U/A) and the SS group (22.73 ± 5.58 U/A) (p<0.0001). The SS group showed DI values higher than in the AA group, but the difference was not significant (p> 0.05). The mean dose of HU and the Length of HU treatment did not affect the DI values (Table IV).

**DISCUSSION**

The clinical and laboratory efficacy of HU in the

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**Table II - Influence of treatment with HU on cell viability evaluated by exclusion assay with Trypan Blue dye in neutrophils from patients with sickle cell anaemia**

<table>
<thead>
<tr>
<th>Group</th>
<th>Viable (%)</th>
<th>Non-viable (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (n=52)</td>
<td>95.8±0.29</td>
<td>4.2±0.33</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>SS (n=20)</td>
<td>93.7±0.34a</td>
<td>6.2±0.35</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>SSHU (n=21)</td>
<td>95.0±0.42</td>
<td>5.0±0.42</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>

AA: Control group (healthy individuals), SS: patients with sickle cell anaemia not treated with HU; SSHU: patients with sickle cell anaemia treated with HU. Results were expressed as mean ± SEM. ANOVA followed by Tukey post test. a p<0.05 versus AA group.

**Table III - Influence of treatment with HU in the parameters of cytotoxicity in neutrophils from patients with sickle cell anaemia**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AA (n=52)</th>
<th>SS (n=20)</th>
<th>SSHU (n=21)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH release (U/L)</td>
<td>6.71±0.68</td>
<td>10.25±1.21</td>
<td>5.59±0.37</td>
<td>0.0003</td>
</tr>
<tr>
<td>MTT reduction (% de viable cells)</td>
<td>56.80±3.20</td>
<td>35.14±2.32</td>
<td>50.59±3.64</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

AA: Control group (healthy individuals), SS: patients with sickle cell anaemia not treated with HU; SSHU: patients with sickle cell anaemia treated with HU. Results were expressed as mean ± SEM. ANOVA followed by Tukey post test. a p<0.05 versus AA group, b p<0.05 versus SS group.

**Table IV - LDH release, MTT reduction and DNA damage according to length of HU treatment and mean HU dose (SSHU group) (n=21)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LDH release (U/L)</th>
<th>MTT reduction (% de viable cells)</th>
<th>DI values (U/A)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean dose of HU (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20.0 mg/kg HU (15)</td>
<td>6.18±0.68</td>
<td>0.289</td>
<td>51.60±8.43</td>
<td>0.825</td>
</tr>
<tr>
<td>≥20.0 mg/kg HU (6)</td>
<td>5.32±0.44</td>
<td></td>
<td>49.90±3.18</td>
<td>28.67±7.39</td>
</tr>
<tr>
<td>Length of HU treatment (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20months (8)</td>
<td>5.88±0.64</td>
<td>0.925</td>
<td>44.81±3.44</td>
<td>0.220</td>
</tr>
<tr>
<td>&gt;20months (13)</td>
<td>5.81±0.44</td>
<td></td>
<td>51.12±3.56</td>
<td>59.0±6.39</td>
</tr>
</tbody>
</table>

Results were expressed as mean ± SEM. Student t-Test. * p<0.05.
treatment of SCA has been convincingly demonstrated, with a decrease in morbidity and mortality with HU use, coupled with the modest short-term toxicity profile and ease of once daily oral administration, HU is an ideal treatment option (Ware, 2010). These consistent results leave little question as to the clinical benefits of HU therapy for adults with SCA. However, the long-term efficacy and safety of HU use for patients with SCA remains one of the most critical unanswered questions (Brawley et al., 2008), and not much is known about the effects of this medicament on neutrophils and on the functionality of these cells, since the majority of the studies found in the literature are related to red blood cells.

The results of this study showed that patients with SCA not treated with HU showed significantly lower cell viability, both the cellular viability assay by Trypan Blue dye as well as the MTT assay, and increased LDH release compared to the control group (AA). Similar results are found in studies with erythrocytes of SCA patients, revealing an increase in plasma levels of LDH of patients when compared to healthy individuals (Kato et al., 2006; Cançado et al., 2009), and this increase in enzymes is positively associated with an increased risk that patients will suffer from priapism, pulmonary hypertension, cutaneous ulcers in the lower limbs, resistance to NO or even early death (Kato et al., 2006; Steinberg, 2008; Rees, Gibson, 2011). There was no significant difference of these variables between the patients treated with HU and the control group (AA). These results suggest that neutrophils from patients with SCA not treated with HU do have damage of cellular integrity and respiratory metabolism, and that HU did not have any cytotoxic effect on patients’ neutrophils when investigated using these testing models. However, a cytoprotective action was revealed when compared to the AA group and the SS patients. It is also possible that the oxidative stress and generalized inflammation of severe untreated SCA produces a mild cytotoxic effect, and HU therapy may help to improve this process.

To evaluate the DNA damage index by comet assay, it was observed that the SCA patients not treated with HU had DI values higher than the AA group, but with no significant difference. No correlation was observed between the DI values with the HbF parameters and the total white blood cell count in the groups studied. Some studies show conflicting results, suggesting that mutagenic or carcinogenic effects are induced by the disease itself rather than by HU therapy (Schultz, Ware, 2003; Segal et al., 2008). For example, in a retrospective survey by the International Association of Sickle Cell Nurses and Physician Assistants, 52 cases of cancer were identified in a cohort of more than 16,000 patients with SCA; almost all occurred in the pre-HU stage; only three patients had previous HU exposure; among children with SCA, 21 reported cancers included leukemia (n=7), Wilms tumor (n=5), lymphoma (n=3), and six other solid tumors; only one patient with acute lymphoblastic leukemia had previous HU exposure (Schultz, Ware, 2003).

There are conflicting reports regarding the DNA-damaging potential of HU in exposed humans. Some studies have shown that HU is genotoxic (Flanagan et al., 2010; Juul et al., 2010; Santos et al., 2011) while other studies suggest that HU has low mutagenicity in vivo (Stricker et al., 1986; Dawkins et al., 1997; Montalembert et al., 1999; Hanft et al., 2000; Montalembert, Davies, 2001; Moschovi et al., 2001). In this study, patients with SCA treated with HU had significantly elevated DI values when compared with the patients with SCA who were not treated with HU. Furthermore, the DI values were significantly higher in patients treated for longer periods (>20 months). Similar results were found in two previous studies that evaluated the DNA Damage Index in total peripheral white blood cell counts of SCA patients treated with HU (Friedrich et al., 2008; Rocha et al., 2012). Taken together, these studies provide evidence for measurable genotoxicity from HU exposure in patients with SCA, but little evidence to support cumulative mutagenicity or carcinogenic potential.

CONCLUSION

Although HU presents numerous positive responses, more studies on the issue of safety are essential, including
the optimal dosage, the duration of use and the age of the patient, among other factors. Currently SCA is characterized as a chronic inflammatory disease where neutrophils initiate leukocyte adhesion to blood vessel walls, contributing thus to the development of inflammatory and vaso-occlusive processes and to the severity of the disease. Despite evidence of the essential involvement of neutrophils in the clinical modulation and the pathophysiological aspects of the disease, the mechanism by which HU modulates this effect is not fully elucidated. This study demonstrates that treatment with HU does not exert a cytotoxic effect on the neutrophils of patients with SCA, and HU may even be able to promote a protective effect on these cells, however there is the risk of DNA damage associated with exposure for longer periods of time. The monitoring of patients with SCA is important, since the data on the cytotoxic and genotoxic risks of HU remain inconclusive. More research is needed to clarify the risks of HU therapy in patients with SCA.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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