Effects of oxidative stress on red blood cell rheology in sickle cell patients

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Summary

Sickle cell anaemia (SS) and sickle cell-haemoglobin C disease (SC) patients exhibit severe red blood cell (RBC) rheological alterations involved in the development of several complications. The contribution of oxidative stress in these haemorheological abnormalities is still unknown. We compared RBC reactive oxygen species (ROS) and glutathione (GSH) content, and the haemorheological profile of SS (n = 11), SC (n = 11) and healthy subjects (n = 12) at baseline and after in-vitro treatment with t-butyl hydroperoxide (TBHP). We showed: (i) higher RBC ROS content in SS and SC patients, with the highest level observed in SS patients; (ii) lower RBC GSH content in sickle syndrome patients, especially in SS patients; (iii) TBHP increased RBC ROS production and decreased RBC GSH content in all groups; (iv) TBHP decreased RBC aggregation and increased the strength of RBC aggregates in all groups but the increase in RBC aggregates strength was greater in sickle cell patients; (v) TBHP decreased RBC deformability in the three groups but with a higher magnitude in sickle cell patients. These data suggest that RBCs from sickle cell patients have an exaggerated response to oxidative stress, which is accompanied by a profound abnormal haemorheological profile, with greater alterations in SS than in SC patients.

Keywords: sickle cell disease, oxidative stress, haemorheology.

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Patients with sickle cell anaemia (SS) and sickle cell-haemoglobin C disease (SC) present severe red blood cell (RBC) rheological alterations, such as reduced RBC deformability and decreased RBC aggregation but increased RBC aggregates strength (Tripette et al, 2009). A reduction in RBC deformability in SS patients is associated with the occurrence of leg ulcers (Connes et al, 2013) and glomerulopathy (Lamarre et al, 2014) and could also be involved in the development of cerebral vasculopathy (Connes et al, 2013). RBC aggregation abnormalities and, more specifically, increased RBC aggregates strength, are associated with an increased risk for acute chest syndrome in both SC and SS patients (Lamarre et al, 2012) and glomerulopathy in SS patients (Lamarre et al, 2014). Indeed, these RBC rheological alterations play a critical role in the development of various acute and chronic complications but the mechanisms at the origin of the large clinical inter-individual variability have not been addressed. The involvement of oxidative stress has recently been suggested (Connes et al, 2014).

Oxygen free radicals have been demonstrated to damage RBCs from healthy donors by decreasing their deformability and aggregability, and by increasing the strength of RBC aggregates (Baskurt et al, 1998). RBCs from SS patients have been reported to generate twofold greater amounts of superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (·OH) than healthy RBCs (Rice-Evans et al, 1986; Hebbel et al, 1988; Hebbel, 1991; Sheng et al, 1998; Aslan et al, 2000; Klings & Farber, 2001; Akohouse et al, 2007). Haemoglobin S (HbS) auto-oxidation processes and recurrent HbS polymerization/depolymerization events that promote intermittent vaso-occlusive episodes and reperfusion injury are a major source of enhanced reactive oxygen species (ROS) production in sickle cell disease (Sheng et al, 1998; Banerjee & Kuypers, 2004). The increased pro-oxidant generation in SS patients results in excessive antioxidant consumption and thus decreased antioxidant capacity (Banerjee & Kuypers, 2004; Reid et al, 2006; Chaves et al, 2008). A recent study reported a negative correlation between oxidative stress...
and sickle RBC membrane damage (Barodka et al., 2014). However, the relationship between the increased oxidative stress of sickle RBCs and their abnormal rheological properties is still poorly documented.

The aim of the present study was to test the effects of t-butyl hydroperoxide (TBHP), a strong oxidant agent, on the rheological properties of RBCs from SS and SC patients and healthy subjects (AA). We hypothesized that TBHP should impair RBC rheology in the three populations but that the alterations could be greater in sickle cell patients.

Materials and methods

Preparation of blood samples

Blood from 11 SC patients, 11 SS patients and 12 healthy controls (AA) were taken into EDTA tubes. The SS and SC patients recruited are regularly followed by the Sickle Cell Unit of the Academic Hospital of Pointe-à-Pitre (Pointe-à-Pitre, Guadeloupe). All participants were aged ≥18 years old. Sickle cell patients were in clinical steady state at the time of the study (i.e., without vaso-occlusive crisis, acute medical complication within the last month or blood transfusion/plebotomies within the last 3 months). Blood was washed two times with phosphate-buffered saline (PBS)-glucose buffer and re-suspended in autologous plasma (Hardeman et al., 2001). Erythrocytes treatment with TBHP 0-3 mmol/l for 20 min at 37°C was chosen as the condition resulting in increased intracellular ROS production, decreased glutathione (GSH) level and no detectable haemolysis. At the end of the incubation (TBHP and CONT conditions), aliquots of the RBC suspensions were taken for determination of ROS and GSH content. The remaining RBC suspension was washed with PBS-glucose buffer and re-suspended in autologous plasma at haematocrit 40% to perform haemorheological measurements. This study is part of the ‘STRESS’ project, which was conducted in accordance with the guidelines set by the Declaration of Helsinki and was approved by the Regional Ethics Committee (CPP Sud/Ouest Outre Mer III, Bordeaux, France, registration number: 2012-A00701-42).

Measurements of RBC ROS and GSH contents

ROS production was measured in RBCs using the membrane permeable probe 2,7-Dichlorofluorescin-diacetate (DCFH-DA; Sigma Aldrich). This probe, once entered into the RBC, is cleaved off by cellular esterases under a non-fluorescent form that is further oxidized by the intracellular ROS, producing the fluorescent 2,7- Dichlorofluorescein molecule (DCF; Sigma Aldrich). Briefly, 1 ml of RBC suspension was washed three times with the PBS-glucose buffer. Then, 100 μl of suspension at 2% haematocrit was mixed with 1900 μl of PBS-glucose buffer, and incubated with DCFH-DA (400 μmol/l) in darkness, for 30 min at 37°C. After incubation, the RBC suspension was washed two times with PBS-glucose buffer, the packed cells were lysed with 1 ml of ice-cold Tris buffer (10 mmol/l Tris/Triton 0.1%, pH 7-4) and then incubated for 30 min in darkness at room temperature. After 30 min incubation, the fluorescence of all samples was read (excitation wavelength: 485 nm and emission wavelength: 538 nm) (Mandal et al., 2005). The results were calculated based on a pre-established standard curve (0-01–10 000 nmol/l) prepared with DCF.

GSH levels were determined using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB; Sigma Aldrich) following the method described by Ellman (1959). Briefly, 50 μl of packed RBC, washed with PBS-glucose buffer, were lysed with 1-25 ml of cold water. Proteins were precipitated by adding 10% trichloroacetic acid (TCA). After centrifugation, one volume of supernatant was added to four volumes of 0.5 mol/l Tris buffer pH 8.2 and DTNB 0.2 mmol/l. After 30 min of incubation in the dark, the absorbance was read at 412 nm.

Determination of RBC aggregation properties

RBC aggregation properties were determined at 37°C by laser backscatter versus time, using the Laser-assisted Optical Rotaional Cell Analyser (LORCA; RR Mechatronics, Hoorn, The Netherlands), after adjustment of the haematocrit to 40% with autologous plasma (Hardeman et al., 2001; Baskurt et al., 2009). Blood was inserted into the Couette system of the LORCA and, after a short period of high shearing (800/s) to dissociate pre-existing RBC aggregates, shearing was stopped abruptly and the changes in laser backscatter intensity was monitored for 2 min (syllectogram) by a photodiode sensor incorporated in the LORCA. The amplitude and the half-time of the syllectogram are then used to calculate the RBC aggregation index. Then, the RBC disaggregation threshold (i.e., RBC aggregates strength) was determined using a re-iteration procedure (Hardeman et al., 2001): seven separate pre-defined shear rates between 7.5/s and 800/s were applied on the RBC suspension, with or without alternating disaggregation shear rate, to locate the minimal shear rate needed to prevent RBC aggregation.

Ektacytometry

RBC deformability was determined at 37°C and 30 Pa by laser diffraction analysis (ektacytometry), using the LORCA. The system has been described elsewhere in detail (Baskurt et al., 2009). Briefly, 25 μl of prepared blood suspension was...
mixed with 5 ml polyvinylpyrrolidone (PVP; viscosity = 30 cP; RR Mechatronics) and sheared into the Couette system made of glass. The diffraction pattern is analysed by the computer and the elongation index is calculated. An increase of the elongation index indicates greater RBC deformability.

**Statistical analyses**

A two-way ANOVA with Newman–Keuls test for post hoc comparisons was used to compare the three groups and test the effects of TBHP on RBC ROS and GSH contents, as well RBC rheological properties. Statistical significance was determined by $P$ value <0.05. Analyses were conducted using **STATISTICA** (Version 8.0; StatSoft, Tulsa, OK, USA) and data were reported as mean ± standard deviation.

**Results**

**RBC ROS and GSH contents**

RBC ROS content was lower in the AA group compared to both the SC and SS groups. Although not significant, the SS group tended to have higher ROS levels than SC patients ($P < 0.1$). TBHP increased ROS production in the three groups (Fig 1). RBC GSH level was higher in the AA and SC groups than in SS patients (Fig 2). TBHP treatment induced a decrease in RBC GSH content all three groups.

**RBC aggregation properties and deformability**

While the RBC aggregation index was not significantly different between the three groups (Fig 3), both SS and SC patients had a higher RBC disaggregation threshold (i.e. RBC aggregates strength; Fig 4) than the AA group. TBHP decreased RBC aggregation and increased the strength of RBC aggregates in the three groups. The percent of increase of RBC disaggregation threshold was 33%, 42% and 48% in the AA, SC and SS group, respectively.

![Fig 1. Effects of RBC treatment with 0-3 mmol/l TBHP on RBC ROS content.](image1)

![Fig 2. Effects of RBC treatment with 0-3 mmol/l TBHP on RBC GSH content.](image2)

![Fig 3. Effects of RBC treatment with 0-3 mmol/l TBHP on the RBC aggregation index.](image3)
RBC deformability was decreased in the two sickle cell groups compared with the AA group but the reduction was more severe in the SS than in the SC group (Fig 5). TBHP decreased RBC deformability in the three groups but the decrease was greater in SS (−21%) and SC (−16%) patients compared to the AA group (−6%).

Discussion

We showed that baseline RBC ROS content was higher in sickle patients compared to healthy subjects, with the highest RBC ROS content observed in SS patients, and that baseline RBC GSH content was lower in sickle cell patients compared to healthy subjects, especially in SS patients. TBHP treatment increased RBC ROS production and decreased RBC GSH content in healthy subjects, SS and SC patients. Concomitantly, TBHP decreased RBC aggregation and increased the strength of RBC aggregates in the three groups but the increase in RBC aggregates strength was greater in sickle cell patients. TBHP also decreased RBC deformability in the three groups but the magnitude of the decrease was higher in sickle cell patients.

TBHP is metabolized by GSH peroxidase and has been demonstrated to be responsible for a reduction in GSH level in healthy RBCs (Rohn et al., 1993). In addition, TBHP may cause RBC fragmentation (Chaves et al., 2008; Lisovskaya et al., 2009) and a reduction in RBC deformability (Chen et al., 1991), depending on the concentration used (ranging from 0·1 mmol/l up to 3 mmol/l). In agreement with an earlier study, (Rohn et al., 1993) we observed a significant decrease in GSH level and an increase in ROS concentration in normal RBCs as well as in sickle RBCs in the presence of as little as 0·3 mmol/l TBHP. Given that this concentration of TBHP did not cause haemolysis (data not shown), all treatments of the RBCs were performed in the presence of TBHP 0·3 mmol/l.

The present study confirmed previous studies showing differences in RBC rheological properties between AA, SC and SS subjects (Tripette et al., 2009; Waltz et al., 2012a,b), with SS patients having the greatest alterations. The effects of TBHP observed in healthy subjects (i.e., decreased RBC deformability and RBC aggregation, and increased RBC aggregates strength) are in agreement with previous findings (Chen et al., 1991; Baskurt et al., 1998). Interestingly, under these oxidative conditions, the rheological profile of RBCs from healthy subjects mimics that of untreated sickle RBCs. These data suggest that the abnormal RBC rheological profiles observed in SS and SC individuals could be partly related to the increased oxidative stress as demonstrated by the increased RBC ROS content and decreased RBC GSH level at baseline conditions.

The additional effects of oxidative treatment on the RBC rheology of sickle cell patients were unknown until now. Although treatment of RBCs with TBHP induced a significant oxidative stress in the three groups, this oxidative stimulus caused a haemorheological response of different magnitude between sickle cell patients and healthy subjects. Indeed, the SS group, and to a lesser extent the SC group, exhibited a greater decrease of RBC deformability and a
higher increase of RBC aggregate strength compared to healthy individuals. This observation could be explained by the higher ROS content and the lower anti-oxidant capacity observed in baseline condition in RBCs from sickle cell patients compared to RBCs from healthy subjects. These abnormal sickle RBC rheological properties might result from the well-known damaging processes induced by ROS which affect RBC membrane lipids and proteins (George et al., 2010). It has been previously shown that oxidative stress causes the generation of methaemoglobin and its denaturation that promotes cytoskeleton rearrangement and band 3 clustering as well as lipid peroxidation and loss of lipid asymmetry (Ferru et al., 2011).

These RBC rheological abnormalities may impair vascular function and microcirculation. For example, Baskurt et al. (2004) demonstrated that a decrease of RBC deformability in the muscle microcirculation resulted in a significant rise of vascular resistance. In addition, increased RBC aggregates strength is highly suspected to increase vascular resistance into the microcirculation, particularly at the pre-capillary level where RBC aggregates need to be fully dispersed before entering into the capillaries (Lamarre et al., 2014; Tripette et al., 2009). Consequently, these alterations in RBC rheology could contribute to the development of several acute and chronic sickle cell anaemia complications, as suggested by Connes et al. (2014).

In summary, the present study demonstrated that RBCs from sickle cell patients have an exaggerated response to oxidative stress compared to RBCs from healthy subjects because of the presence of a deficient anti-oxidant system. The profound RBC rheological changes observed in sickle cell patients (i.e., decreased RBC deformability and increased RBC aggregates robustness) may play a major role in sickle cell disease pathophysiology.

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Authorship

PC, XW, RH, MR, NL and MDHD designed the research study; XW, RH, PM performed the experiments; PC, XW, RH, PM, MDHD analysed the data; PC, XW, RH, MR and MDHD wrote the paper, RH, XW, PM, MR, NL, PC and MDHD read and approved the final version of the manuscript.

Conflict of interest

No conflict of interest.

References


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