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Red blood cell mechanical sensitivity improves in chronically transfused sickle-cell patients following prolonged, sub-hemolytic shear exposure.

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Abstract

Background: Sickle cell disease (SCD) is a genetically-inherited hemoglobinopathy where deoxygenated hemoglobin S polymerizes, leading to stiff red blood cells (RBC) and inefficient microcirculatory blood flow. Transfusion therapy acts as primary and secondary prevention of ischemic stroke in SCD. Whether blood transfusion alters the mechanical sensitivity of RBC to prolonged sub-hemolytic shear stress (shear) is unknown. We hypothesized that individuals with SCD undergoing chronic blood transfusion would have improved sensitivity to shear, compared with patients not on transfusion therapy.

Methods and Materials: Blood suspensions from individuals with SCD not receiving ($n = 15$) and receiving ($n = 15$) chronic simple-transfusion were conditioned to shear (1, 4, 16, 32, 64 Pa) for various durations (1, 4, 16, 32, 64 s), and then deformability of RBC was immediately measured. Healthy young Controls ($n = 15$) were included for reference. A surface-mesh was interpolated using the data to determine the effect of blood transfusion on mechanical sensitivity of RBC.

Results: There was impaired RBC deformability to prolonged supraphysiologic shear in both SCD groups; however, mechanical sensitivity actually improved in transfused patients when exposed to prolonged physiologic shear. Further, in the transfused SCD patients, the threshold above which subhemolytic damage occurs was similar to Control.

Conclusion: We found that chronic transfusion therapy normalizes the mechanical sensitivity threshold above which RBC subhemolytic damage occurs following prolonged shear exposure in

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Conflict of Interest Statement

All authors state that there are none to declare.

SCD. An important and novel finding in transfused SCD patients was the improvement in RBC deformability in response to prolonged shear exposure over the physiologic range.

Keywords

cell fragility; erythrocyte; high shear; preconditioned

Introduction

In certain regions of the world sickle cell disease (SCD) is still fatal within the first years of life; however, increased lifespan in developed countries has elevated the incidence of chronic vascular disease and end-organ damage¹. SCD stems from a single point modification in the *beta-globin* gene resulting in a mutant beta-globin chain that, when combined with alpha globin, forms hemoglobin (Hb) S². When red blood cells (RBC) traverse the capillary network Hb S releases oxygen to the tissue and transitions from the R-state to the T-state, resulting in Hb S polymer formation. Polymerization is reversible and thought to be ongoing, even in discoid RBC^{3–5}. Polymerization results in decreased RBC deformability of discoid RBC, and in some RBC, overt sickling occurs⁶. Cyclic polymerization-depolymerization events decrease the cell's capacity to dynamically change shape in response to mechanical stress (i.e., dynamic change in RBC deformability). This process utilizes energy, and with chronic cycling of Hb polymers, mechanical damage accumulates in the cell ultimately resulting in hemolysis⁷.

Acute and chronic transfusions of AA RBC are disease modifying in SCD, providing both chronic prevention of and acute therapy for complications associated with the disease. Acute transfusion may be used during splenic sequestration, aplastic crises, acute chest syndrome, and stroke⁸. Chronic transfusion therapy serves as both primary and secondary prevention of stroke but may also be used for unremitting pain crises and pulmonary hypertension. Despite the benefits of blood transfusion, it has been reported over several decades that blood storage increases the fragility of RBC and impairs cellular deformability^{9–11}. This may predispose transfused AA RBC to mechanical damage, and thus depending on the percentage of AA versus SS RBC, cell mechanics in transfused patients may still be suboptimal.

While exposure of RBC to shear stress facilitates cellular deformation and aligns cells with fluid flow, exposure to shear well-above the peak levels within the human circulation of ~10–15 Pa¹² is damaging to cells. Recent studies demonstrate that supraphysiological shear – as induced by rotary blood pumps – caused sublethal trauma^{13,14} to RBC even when levels were below that required to induce hemolysis¹⁵. While historically rare, chronic cardiovascular complications occur due to improved survival and thus patients with SCD may require cardiac surgery requiring cardiopulmonary bypass. It has indeed been reported that individuals with SCD received extracorporeal membrane oxygenation treatment in the management of acute chest syndrome^{16,17}, and more recently cardiac dysfunction¹⁸, despite the associated high shear environment of these blood circuits. Little is known about the effect of subhemolytic shear stress – that is, shear stress levels that are supraphysiological yet below that which induces hemolysis – on RBC from individuals with SCD who are chronically transfused. Given this gap in knowledge about the effects of prolonged

subhemolytic shear stress on RBC, we investigated how varied duration and magnitude of shear stress influences RBC mechanical sensitivity in individuals with SCD on chronic blood transfusion therapy. Our hypothesis was that RBC from individuals with SCD undergoing chronic blood transfusion would have improved mechanical sensitivity following exposure to prolonged shear stress, when compared with non-transfused SCD patients.

Materials and Methods

Subjects and sample preparation

Patients with SCD, both non-transfused and chronically transfused, were enrolled from the hematology clinic and transfusion center at Children's Hospital Los Angeles. Patients on chronic transfusion therapy were receiving simple transfusion every two-to-three weeks with the goal to regulate Hb S to <30% of total hemoglobin. Control subjects were enrolled from a convenience sample at Griffith University. All subjects volunteered to provide a blood sample after informed consent was obtained. Controls were free from known disease and were not on any medications. Blood was drawn using a 21 G needle from a peripheral vein into heparinized vacuum tubes, well mixed, then placed on a slowly rotating roller until analyses. Blood collection was completed within 90 s of tourniquet application, and all experiments were done within 4 h of sampling. Blood collection methods were consistent with recommendations for hemorheological assessment to minimize cell disruption and to standardize shear forces on samples¹⁹. The study protocol was consistent with The Code of Ethics of the World Medical Association (Declaration of Helsinki), and was reviewed and approved by the Institutional Review Boards at Children's Hospital Los Angeles and Griffith University, Australia.

Sample preparation

The experimental protocol for each subject (see below) required the use of 27 RBC suspensions for all measurements to be completed; these were prepared in small batches (five samples per batch) immediately prior to analysis. The suspensions were prepared by diluting 1:200 whole blood in a viscous, isotonic solution of polyvinylpyrrolidone (PVP; 360,000 Da, dissolved in 0.1 mol·L⁻¹ phosphate buffered saline; typical viscosity = 28.1 mPa·s at 37°C, pH = 7.39, 289 mOsmol·kg⁻¹; Mechatronics Instruments B.V., Zwaag, The Netherlands). All samples were analyzed within 10 min of preparation.

Experimental design

The experimental design involved three phases: (1) determination of *unsheared* RBC deformability to provide a baseline; (2) a "conditioning" shearing period at a specific level and duration of shear stress, and; (3) the subsequent determination of RBC deformability immediately following the conditioning period. This protocol has been described in detail previously¹⁴.

RBC deformation in response to an applied fluid shear stress for unsheared cells was determined using a laser-diffraction ektacytometer system (see below, Section "RBC deformability measurements"); these data provided the baseline values of RBC deformability. Subsequently, freshly-prepared RBC suspensions were exposed to a

conditioning stimulus that consisted of a given level of shear for a specific duration. The magnitudes of shear were 1, 4, 16, 32, and 64 Pa. Each shear level was used with a new sample for a given duration, where the durations were 1, 4, 16, 32, and 64 s. Thus, each assay consisted of a baseline measurement of unsheared cells (i.e., baseline), and RBC suspensions subjected to 25 conditioning stimuli: five levels of shear and five exposure times (i.e., 1 Pa x 1 s, 1 Pa x 4 s... 64 Pa x 32 s, 64 Pa x 64 s) in a randomized order. Baseline measurements on unsheared RBC were repeated at the end of each experiment to determine whether *ex vivo* ageing influenced RBC deformability.

RBC deformability measurements

RBC deformability was measured using a concentric cylinder ektacytometer (LORRCA MaxSis, Mechatronics Instruments B.V., Zwaag, The Netherlands) operating at $37 \pm 1^\circ\text{C}$. Dilute suspensions of RBC in the viscous PVP solution were gently transferred into the gap between the cup and bob of the instrument and air bubbles were cleared while the sample was slowly sheared (0.1 rev s^{-1} ; shear 0.2 Pa) to allow temperature equilibration. The RBC suspension was then subjected to various shear stress ranging from 0.3 Pa to 50 Pa. A low-power laser beam (670 nm, 4 mW) projected from the bob, through the sample and cup, and onto a small projection screen. The laser beam generated a diffraction pattern that was circular for cells at rest and became progressively ellipsoidal as RBC deformed with shear stress. Diffraction patterns were captured by an integrated closed couple device camera and analyzed in real-time by fitting an ellipse to the region of interest. An elongation index (EI) of the diffraction pattern was calculated: $\text{EI} = (\text{length} - \text{width})/(\text{length} + \text{width})$, based on the geometry of the ellipse.

Hematologic Measures

Heparinized samples were also analyzed to evaluate hematocrit, Hb levels, white blood cell (WBC) fractions and platelet count. Electrophoresis was used to determine Hb fractions (i.e., Hb A, S, F) in individuals with SCD. Markers of hemolysis included concentrations of plasma free Hb, lactate dehydrogenase, and bilirubin.

Data Analysis

Data generated by the ektacytometer were analyzed using a commercial analytical software program employing a non-linear regression approach (Prism, GraphPad Software Inc, Release 7.02, USA). RBC previously exposed to high stresses for longer periods exhibited atypical elongation index (EI) data when deformability was measured below 0.94 Pa, and hence using such EI values yielded non-sigmoidal EI-shear stress curves and spurious curve-fit parameters. This has been described elsewhere as sickle RBC tumbling at low shear, creating a “cross-shape” pattern that is inaccurate for EI determination²⁰. All data for regression analyses were thus truncated and curves were only fit to EI-shear stress data over the range of 0.94–50.0 Pa. Non-linear regression²¹ yielded the following indices: (1) the maximum theoretical EI at infinite shear (EI_{max}); (2) the shear stress required for half of EI_{max} ($\text{SS}_{1/2}$); the ratio $(\text{SS}_{1/2})/(\text{EI}_{\text{max}})$ was used to scale for changes of maximal deformation. Note that decreased RBC deformability yields an increased $\text{SS}_{1/2}$, a decreased EI_{max} and hence an increased ratio.

A Mechanical Sensitivity (MS) parameter was calculated for all samples using the formula previously reported²²:

$$MS(\%) = \frac{\frac{SS_1 / 2^n}{EI_{max}^n} - \frac{SS_1 / 2^{BL}}{EI_{max}^{BL}}}{\frac{SS_1 / 2^{BL}}{EI_{max}^{BL}}} \times 100$$

where n represented the specific level-duration shear exposure, and BL represented the unsheared baseline value.

Surface plots were produced by creating an x,y,z matrix of the mean MS data for all subjects, where z was MS, and x and y represented the duration and magnitude of shear exposure, respectively (Origin, OriginLab Corp., Release 8, Northampton, MA). Thin-plate splines were used to interpolate the raw x,y,z matrix, and a surface plot of the matrix facilitated visual identification of the subhemolytic threshold. The subhemolytic threshold was conservatively considered to occur at the +95% confidence interval above the baseline mean for MS. That is, if an MS value was >95% of the confidence interval of the baseline mean, this value was considered to be *outside* the expected range and thus indicated impairment of RBC deformability (see Figure 3). All MS values between -95% and +95% confidence intervals of the baseline mean were considered “normal” and thus not affected by the conditioning protocol.

Various models were pilot-tested to describe the relation between the level of shear and exposure duration: two- and three-component exponentials had poorer predictive power (r^2) than a single-exponential decay curve fit, while 2nd, 3rd, 4th, 5th and 6th order polynomial curve fits either did not fit the data or were again poorer fits (r^2) of the raw data. Consequently, the most robust fit of the level of shear stress and exposure duration was found using the monoexponential equation using nonlinear least-squares regression (Prism, GraphPad Software Inc, Release 7.02, USA).

Statistical analysis

Results are reported as mean $\pm$ standard error. Normality of the data was confirmed using the D’Agostino and Pearson omnibus normality test (Prism, GraphPad Software Inc, Release 7.02, USA). Data for each condition were compared using an appropriate ANOVA, with repeated measures when necessary, to determine whether significant differences in the means existed (SPSS Inc, Release 20.0, USA). Post hoc comparisons were analyzed using Tukey’s corrections when appropriate. Significance was determined at an alpha level of 0.05.

Results

Patient characteristics and hematological profile

The patient demographics, anthropometric data and hematological measures are reported in Table 1. We enrolled 30 patients with SCD (15 chronically transfused and 15 non-

transfused: 43% were female; age was 17 ± 6 yr). We also enrolled 15 healthy subjects: 20% were female; age was 20 ± 1 yr. The SCD groups presented with typical hematology variations when compared with Controls: they were anemic and had evidence of chronic inflammation and chronic hemolysis. The two SCD groups were well matched for height and body mass. We only measured pre-transfusion lab values, and they were not significantly different from non-transfused patients for WBC, platelets, RBC, or reticulocytes. Total Hb was not different between SCD groups; however, significant differences were detected, as expected, for Hb variants: non-transfused patients had significantly elevated Hb S and Hb F, and significantly decreased expression of Hb A, when compared with the transfused group in the present study (all $p < 0.001$). The non-transfused group presented with 10% larger RBC (i.e., increased mean corpuscular volume) when compared to chronically transfused patients, which is likely due to hydroxyurea use ($p = 0.003$). No significant differences were observed between SCD groups for markers of hemolysis: plasma free Hb and lactate dehydrogenase were similar between groups, although bilirubin concentration was significantly higher in transfused when compared with non-transfused patients ($p = 0.013$).

RBC deformability was improved in the chronically-transfused group

Red blood cell deformability for each group is illustrated in Figure 1. The RBC deformability response to shear was typically sigmoidal (Figure 1A), with increased deformation observed upon application of higher shear levels. When compared with typical values for non-SCD individuals (Controls), both transfused and non-transfused patients demonstrated impaired RBC deformability. Impaired RBC deformability in the transfused group was partially ameliorated compared to the non-transfused group ($F = 7.715$; $p = 0.010$). Specifically, Elongation Indexes were increased for transfused individuals at shear stresses of 2.81 Pa ($p = 0.029$), 5.00 Pa ($p = 0.009$), and 8.89 Pa ($p = 0.035$), when compared with the non-transfused group. The $SS_{1/2}:EI_{max}$ parameter (Figure 1B), a simplified and summative metric of RBC deformability²¹, was also improved for transfused patients ($p = 0.003$).

Effects of prior exposure to shear stress on RBC deformability

The RBC deformability (i.e., $SS_{1/2}:EI_{max}$) response to varying conditioning shear stress are illustrated in Figure 2 for the SCD groups. The deformability of RBC measured without prior shear stress conditioning before (Pre) and after (Post) the experiment are presented for comparative purposes. For all combinations of conditioning shear and exposure duration, RBC were more deformable in the transfused group, compared with non-transfused. There was no effect of *ex-vivo* aging on RBC deformability (i.e., Pre vs. Post measurements); therefore, all responses we documented were the result of conditioning shear.

In the non-transfused group, conditioning at 1, 4 and 16 Pa for all exposure durations (1–64 s) did not influence deformability. When conditioned for 64 s at 32 Pa, RBC became less deformable ($p = 0.022$). Conditioning at 64 Pa significantly worsened deformability over all exposure durations – 1 s ($p = 0.024$), and 4, 16, 32, and 64 s (all $p < 0.001$, inclusive).

Unique responses were observed following conditioning shear in the transfused SCD group: we found both impaired RBC deformability, and improved RBC deformability, that were dependent upon shear conditioning. Conditioning at 4 Pa (4, 16, 32, 64 s; all $p < 0.001$), 16 Pa (1, 4, 16, 32, 64 s; all $p < 0.01$) and 32 Pa (1, 4, 16, 32 s; all $p < 0.05$) significantly improved RBC deformability in the transfused group. Impaired deformability was also observed, however, in the transfused group but only after conditioning at 64 Pa for 16, 32 and 64 s (all $p < 0.001$).

A surface mesh indicating RBC sensitivity to conditioning shear stress (i.e., Mechanical Sensitivity, MS) is illustrated in Figure 3. The magnitude of MS was greater at all combinations of shear stress and exposure duration in the two SCD groups when compared with Controls, although there was a prominent amelioration in the transfused group. The upper 95% CI for the MS data (solid white line $>+95\%$ CI, Figure 3) was found to occur at +9.0% in non-transfused (Figure 3, *Left*) and +7.9% in transfused (Figure 3, *right*), which indicates significant sublethal mechanical impairments occurs above this point. Critical levels of MS (i.e., $>95\%$ CI) demonstrated an inverse relation between shear stress and duration. Peak MS reached 50% at 64 Pa x 64 s in the non-transfused group, which is ~25% greater than the corresponding value in the transfused group (40% at 64 Pa x 64 s).

The upper 95% CI of the MS (Figure 3) can be described using a monoexponential decay of the shear stress level and exposure duration: the duration-magnitude interactions required to impair RBC deformability (i.e., $>+95\%$ CI) are shown in Figure 4. The curve fits were robust for both groups; non-transfused: $r^2 = 0.988$, standard error of the estimate = 0.98 Pa; transfused: $r^2 = 0.986$, standard error of the estimate = 0.79 Pa. Substantial differences in the y-intercepts were detected, indicating near instantaneous exposure to 70.2 Pa (non-transfused) and 79.8 Pa (transfused) would induce impaired cell mechanics; transfusion thus partially normalized the equivalent value observed in healthy Controls (87.3 Pa). The asymptotic shear stress differed by ~25% between the two SCD groups; specifically, impaired RBC deformability would be induced following prolonged (i.e., infinite) exposure to 32.2 Pa for the non-transfused group, and 42.2 Pa in the transfused group. The equivalent asymptotic shear stress in healthy Controls was 40.1 Pa.

Discussion

We aimed to determine the effects of prolonged shear stress on the MS of RBC by assessing induced changes in cellular deformability – an important determinant of microcirculatory blood flow and tissue oxygenation. We observed that RBC mechanical sensitivity improves after conditioning with physiologic, and moderate supraphysiological, levels of shear stress in Control subjects and SCD patients on chronic transfusion therapy (Figure 2 and 3). No improvements in RBC mechanical sensitivity were observed, however, in a matched group of non-transfused patients. Moreover, the threshold above which subhemolytic damage occurs was normalized in chronically transfused SCD patients (Figure 4), although the absolute cell mechanics at this point were still not completely reflective of non-SCD individuals (i.e., Controls). Collectively, these mechanical sensitivity findings provide new insight into biomechanical improvements to RBC that supersede standard measures of

deformability, which is crucial when evaluating RBC mechanics in flowing systems such as blood pumps and in the human microcirculation.

There are several potential mechanisms underlying the improved RBC deformability following prolonged exposure to physiologic shear stress (Figures 2 and 3) and the subhemolytic threshold above which adverse mechanical changes occur (Figure 4), including: i. dilution of SS RBC with AA RBC; ii. RBC membrane or cytosolic modifications that act to modulate mechanical sensitivity, and; iii. improved plasma environment, that may reflect decreased chronic inflammation and products of hemolysis.

Dilution of SS RBC with AA RBC likely plays an acute role in our findings; however, our experimental setup does not allow us to predict the fraction of SS RBC that must be achieved to maximize the improvement in mechanical sensitivity. Over the short lifetime of RBC in patients with SCD, blood is continuously exposed to varying shear across the vascular network, the level and duration of which are principally determined by dynamic relations between fluid viscosity, flow rate, cell biophysical properties, and vascular geometry. In SCD, RBC are less deformable in general and worsen upon deoxygenation, increasing viscosity and thus local shear stress. SS RBC adhere to the endothelium, which leads to vascular pruning and altered vascular geometry²³, further altering local shear stress. With accumulated shear exposure, SS RBC are particularly susceptible to overt hemolysis, although it is presently apparent that subhemolytic trauma also accumulates, resulting in microparticle formation. RBC microparticle formation is well known in SCD and has been linked to both physiologic and clinical outcome measures²⁴. Therefore, the implications of these data directly relate to a key question in sickle cell disease and transfusion therapy, both acute and chronic: what is the optimal SS RBC fraction required to confer maximum benefit, while inducing the least burden on typically tissue-poor blood banks? Mechanical sensitivity appears to be a potential biomarker that may prove useful in addressing this question.

The mechanism by which cellular deformability actually *improved* following short-term (1–64 s) exposure to shear within the physiological range (i.e., 4–16 Pa; Figure 2) is unclear because this has not been observed previously in SCD and only recently reported in healthy subjects²⁵. Potential mechanisms for these changes include RBC membrane mechanosensitive changes that alter cellular transport of water and calcium, and nitric oxide (NO) produced locally by an NO synthase (NOS) isoform²⁶. RBC-NOS derived NO may have the capacity for maintaining intracellular NO balance and modulating RBC deformability^{27–29}. Intracellular NO has the capacity to bind thiol groups (i.e., S-nitrosylation), and when RBC cytoskeletal proteins – specifically α and β spectrins – are S-nitrosylated, improved cellular deformability has been observed³⁰. It has also been shown that nitrogen oxides may play a role in Gardos channel modification causing the cells to maintain hydration, thus improving RBC deformability²⁷.

Studies exploring such pathways under shear stress have been limited in SCD, and findings on the capacity for RBC-NOS to enhance³¹ or impair³² cellular deformability in these patients are conflicting. These studies have only explored basal RBC-NOS activity, and its correlation with RBC deformability. To our knowledge, no studies have explored shear-

mediated RBC-NOS activation and cellular deformability in a well-controlled dynamic system utilizing SS RBC.

With regard to plasma suspending environment, chronic transfusion therapy has been shown to improve vascular function in medium size arteries and single measures of RBC deformability, but it did not normalize markers of inflammation or hemolysis³³. The STOP study³⁴ was a randomized control trial with plasma haemoglobin levels measured prior to the initiation of transfusion therapy, and then again after one year of chronic transfusion therapy. Patients in the STOP study had significantly elevated plasma haemoglobin levels at a baseline, being 78.7 mg/dL prior to initiation of transfusion therapy. After one year on transfusion therapy, SCD patients achieved a level of 34.2 mg/dL. The plasma free haemoglobin levels we observed (transfused: 28.0 mg/dL; non-transfused: 32.0 mg/dL) are similar to those published in the STOP study following one year on chronic transfusion, although our study was cross-sectional and did not have plasma free haemoglobin measurements prior to the initiation of the transfusion therapy. Our data is nevertheless consistent with previous reports, and indicates that while transfusion is effective for reducing plasma free haemoglobin, it does not normalize to levels comparable with healthy Controls.

The subhemolytic threshold observed in our non-transfused SCD patients was lower (i.e., worse) when compared with our current healthy Control group as well as previous reports in normal RBC¹⁴. Importantly, subhemolytic trauma was observed at shear stress levels well-below that induced by rotary blood pumps used for cardiopulmonary bypass or ventricular assist devices³⁵. In our transfused patients, however, no detectable impairment in RBC deformability was observed until conditioning reached 64 Pa for at least 16 s. Thus, simple transfusion in SCD has the capacity to substantially reduce the susceptibility of RBC to mechanical impairments that result from prolonged shear exposure. This may be important when determining transfusion protocols for surgeries in our surviving population of patients with SCD who require cardiovascular surgery. Considering SCD is listed on the current exclusion criteria for popular ventricular assist devices (e.g., Berlin Heart³⁶), these findings may affect certain strategies for cardiovascular therapies; surgical and otherwise.

While we have found an improvement in RBC mechanical sensitivity with chronic transfusion therapy, our study has some limitations. We are not able to directly link our findings with improved microcirculatory function and tissue oxygenation, nor confirm specific mechanistic explanation for these observations. The cross-sectional nature of this study also limits our ability to understand how this finding changes with time, which is important for our understanding of chronic vascular disease. Our working hypothesis is that dilution of Hb S RBC with healthy RBC results in an *acute* improvement in population RBC deformability and cell mechanics. However, *chronic* improvements are likely due to enhanced intracellular and cell-cell signaling of molecules known to augment deformability.

In conclusion, we found that chronic transfusion therapy normalizes the mechanical sensitivity threshold above which RBC subhemolytic damage occurs following prolonged shear exposure in SCD. An important and novel finding was the improvement in RBC deformability of SCD patients in response to prolonged shear exposure over the physiologic range.

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Figure 1. Red blood cell Elongation Index (i.e., deformability) measured at discrete shear stresses (Panel A) for individuals with sickle cell disease (SCD) and young Controls.

Chronically-transfused patients (○) presented with significantly improved RBC deformability when compared with the non-transfused group (■). Typical RBC deformability for non-SCD individuals is provided as a point of reference (Δ). The shear stress required for half-maximal elongation index (SS_{1/2}) expressed relative to maximal theoretical elongation index (EI_{max}) was significantly impaired in both SCD groups compared with Control; although the transfused group (Panel B) were significantly

improved when compared with the non-transfused group. Significant differences between groups: ***, $p < 0.001$. **, $p < 0.01$.

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Figure 2. Deformability of RBC (expressed as $SS_{1/2}:EI_{max}$) measured immediately after exposure to discrete shear stresses (1, 4, 16, 32, 64 Pa) for specific durations (1, 4, 16, 32, 64 s). We also measured RBC deformability in samples that were not conditioned with shear stress at the start of the study, and again at the end (Pre and Post); this allowed us to determine whether deformability changes were due to shear application or due to *ex-vivo* aging. Data is grouped by whether or not patients had a history of chronic transfusion therapy. Significant differences after conditioning were noted when compared with pre-conditioning values for: †, non-transfused patients, and; ‡, chronically transfused patients.

Figure 3. Mechanical Sensitivity (MS) of RBC is dependent upon the duration and magnitude of shear exposure; presented for healthy Controls (top), and individuals with sickle cell disease not on chronic transfusion (middle) and those on chronic transfusion (lower).

A surface mesh has been overlaid onto raw data indicated as: (1) green, MS less than lower 95% Confidence Interval (CI); (2) light grey, MS greater than lower 95% Confidence Interval (CI), but less than mean; (3) dark grey, MS greater than the mean, but less than the upper 95% CI; (4) red, MS greater than upper 95% CI. Note: colored regions are based on the mean and confidence intervals for each subgroup, and thus analyses are *relative* to each group.

Figure 4. The shear stress and duration of exposure required to impair red blood cell deformability for healthy Controls, non-transfused sickle cell disease patients and chronically-transfused patients.

Solid lines represent the respective exponential curve fits used to describe the relation between shear stress and duration that induced impaired RBC deformability. Shaded regions represent the 95% Confidence Intervals of the respective curve fits.

Table 1.

Physical characteristics and hematological measures

	Control	NTrans	Transfused	
Age (y)	20 ± 1	17 ± 8	18 ± 5	
Sex (m / f)	12 / 3	11 / 4	6 / 9	
Height (cm)	179 ± 6	156 ± 20	157 ± 12	<i>a,b</i>
Body mass (kg)	71.7 ± 9.0	57.5 ± 22.7	56.8 ± 19.1	<i>a,b</i>
Body mass index (kg·m ⁻²)	23.3 ± 1.8	22.5 ± 5.4	22.4 ± 5.6	
Number of transfusions (/yr)	0	0	16 ± 2	<i>b,c</i>
Time since last transfusion (d)	n/a	n/a	24 ± 6	
WBC count (x10 ⁹ ·L ⁻¹)	6.0 ± 1.5	9.1 ± 6.4	12.6 ± 4.5	<i>c</i>
Platelet count (x10 ⁹ ·L ⁻¹)	192 ± 34	362 ± 228	279 ± 142	
RBC count (x10 ¹² ·L ⁻¹)	4.9 ± 0.4	3.0 ± 0.8	3.1 ± 0.4	<i>a,b</i>
Reticulocytes (%)	1.1 ± 0.4	7.5 ± 5.2	10.7 ± 6.1	<i>a,b</i>
Hematocrit (L·L ⁻¹)	43.7 ± 3.1	28.3 ± 5.5	27.4 ± 3.4	<i>a,b</i>
Hb (g·L ⁻¹)	14.8 ± 9.8	9.8 ± 1.9	9.1 ± 1.1	<i>a,b</i>
Mean corpuscular volume (fL)	88.7 ± 4.0	97.4 ± 13.5	88.6 ± 3.0	<i>a,c</i>
Mean corpuscular Hb (pg)	30.2 ± 1.3	33.9 ± 5.7	29.5 ± 1.3	<i>a,c</i>
Hb A (%)	-	3.2 ± 1.2	75.1 ± 13.2	<i>c</i>
Hb S (%)	-	77.3 ± 11.2	20.6 ± 12.0	<i>c</i>
Hb F (%)	-	19.7 ± 12.0	1.7 ± 1.7	<i>c</i>
Plasma free Hb (mg·dL ⁻¹)	-	32.0 ± 32.3	28.0 ± 38.5	
Lactate dehydrogenase (U·L ⁻¹)	-	1012 ± 415	1103 ± 558	
Bilirubin (mg·dL ⁻¹)	-	2.1 ± 1.3	4.1 ± 2.4	<i>c</i>

NTrans: non-transfused patients with sickle cell disease. WBC: white blood cell. RBC: red blood cell. Hb: hemoglobin.

a, non-transfused significantly different from Control*b*, transfused significantly different from Control*c*, transfused significantly different from non-transfused.