Received: 22 February 2021 | Accepted: 27 May 2021

DOI: 10.1111/jth.15407

ORIGINAL ARTICLE

Pathologically stiff erythrocytes impede contraction of blood clots

Valerie Tutwiler[1] | Rustem I. Litvinov[1,2] | Anna Protopopova[1] | Chandrasekaran Nagaswami[1] | Carlos Villa[3] | Eric Woods[4] | Osheiza Abdulmalik[5] | Don L. Siegel[3] | J. Eric Russell[6] | Vladimir R. Muzykantov[7] | Wilbur A. Lam[8] | David R. Myers[8] | John W. Weisel[1]

[1]Department of Cell and Developmental Biology, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA

[2]Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russian Federation

[3]Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA

[4]Max-Planck-Institut für Eisenforschung GmbH Düsseldorf, Düsseldorf, Germany

[5]Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA

[6]Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA

[7]Department of Pharmacology, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA

[8]The Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology & Emory University, Atlanta, Georgia, USA

Correspondence
John W. Weisel, Department of Cell & Developmental Biology, University of Pennsylvania School of Medicine, 421 Curie Blvd, BRB II/III, Room 1154, 19104-6058 Philadelphia, PA, USA.
Email: weisel@pennmedicine.upenn.edu

Funding information
NIH K99/R00HL148646-01 (V.T.), R01-HL135254 (J.W.W.), Bridge grant from the Perelman School of Medicine, the Kazan Federal University Strategic Academic Leadership Program (R.I.L.). Scanning electron microscopy was supported by NIH Shared Instrumentation Grant S10-OD018041 (J.W.W.).

Abstract

Background: Blood clot contraction, volume shrinkage of the clot, is driven by platelet contraction and accompanied by compaction of the erythrocytes and their gradual shape change from biconcave to polyhedral, with the resulting cells named polyhedrocytes.

Objectives: Here, we examined the role of erythrocyte rigidity on clot contraction and erythrocyte shape transformation.

Methods: We used an optical tracking methodology that allowed us to quantify changes in contracting clot size over time.

Results and conclusions: Erythrocyte rigidity has been shown to be increased in sickle cell disease (SCD), and in our experiments erythrocytes from SCD patients were 4-fold stiffer than those from healthy subjects. On average, the final extent of clot contraction was reduced by 53% in the clots from the blood of patients with SCD compared to healthy individuals, and there was significantly less polyhedrocyte formation. To test if this reduction in clot contraction was due to the increase in erythrocyte rigidity, we used stiffening of erythrocytes via chemical cross-linking (glutaraldehyde), rigidifying Wright^{b} antibodies (Wr^{b}), and naturally more rigid llama ovalocytes. Results revealed that stiffening erythrocytes result in impaired clot contraction and fewer polyhedrocytes. These results demonstrate the role of erythrocyte rigidity in the contraction of

Manuscript handled by: Roger Preston

Final decision: Roger Preston, 27 May 2021

blood clots and suggest that the impaired clot contraction/shrinkage in SCD is due to the reduced erythrocyte deformability, which may be an underappreciated mechanism that aggravates obstructiveness of erythrocyte-rich (micro)thrombi in SCD.

KEYWORDS

blood clotting, clot retractions, coagulation, sickle cell disease, thrombosis

1 | INTRODUCTION

Clot contraction results in the volume shrinkage of blood clots and may play a role in processes such as hemostasis, thrombosis, and wound healing.[1,2] The intravital shrinkage of clots and thrombi may have pathogenically important effects, such as modulation of local blood flow past a partially obstructive thrombus and changes of porosity and permeability of thrombi for fibrinolytic enzymes.[3] The extent of compression and densification of a thrombus can determine the likelihood of its mechanical rupture, potentially leading to thrombotic embolization.[4] Several clinical studies have revealed that clot contraction is suppressed in the blood of patients with (pro)thrombotic conditions, such as ischemic stroke,[5] venous thromboembolism,[4] systemic lupus erythematosus,[6] miscarriage,[7] and imminent postoperative thrombosis.[8]

Contractile forces in a blood clot are generated by activated platelets[9,10] and transmitted through the fibrin network, resulting in the platelet--fibrin meshwork accumulating at the periphery of the clot and the erythrocytes being compacted into the core of the clot.[1] When erythrocytes become compressed within the contractile platelet-fibrin envelope, they form a tessellated array and take on a polyhedral shape.[1,11] This shape change resulted in the terminology of polyhedrocytes or piezocytes,[11] and is due to the fact that erythrocytes are highly deformable cells that recurrently experience reversible shape changes due to fluid forces while traveling through the microcirculation. The presence of polyhedrocytes has been observed in *in vitro* clots[1,11] as well as *ex vivo* arterial and venous thrombi, hemostatic clots, and thrombotic emboli from mice and humans.[1,4,12,13,14] The extent and rate of clot contraction is inversely related to the volume fraction of erythrocytes, with the incorporation of more erythrocytes resulting in an increase in resistance to the platelet-generated contractile forces.[15,16] However, it is unknown whether the mechanical properties or deformability of the erythrocytes modulate clot contraction and polyhedrocyte formation. Theoretical modeling of the interplay between the contractile activity of platelets and mechanical resilience of the mass of erythrocytes entrapped in a clot[15] strongly suggests that qualitative changes in erythrocytes' viscoelasticity can play a role in the mechanical remodeling of blood clots and thrombi. If this assumption is correct, it has important pathophysiological implications, because reduced erythrocyte deformability is associated with a number of pathological conditions, such as hypertension, diabetes mellitus, atherosclerosis, and smoking.[17-21] One of the best-known diseases associated with increased erythrocyte rigidity is sickle cell trait or sickle cell disease (SCD).[22-24] SCD is a hypercoagulable state that is associated with vaso-occlusive events in the microcirculation and an increased risk of venous thromboembolism.[25-30]

Essentials

- Platelet-driven blood clot contraction/volume shrinkage can modulate obstructiveness of thrombi.
- Naturally or artificially rigidified erythrocytes reduce the extent of blood clot contraction.
- In sickle cell disease patients, blood clot contraction is impaired due to stiffer erythrocytes.
- Clot-entrapped erythrocytes with increased rigidity suppress contraction and promote thrombosis.

Erythrocytes contain about 27 to 31 pg/cell or 32% to 36% the protein hemoglobin, which is responsible for the exchange of oxygen and carbon dioxide between the blood and tissues.[31] Hemoglobin is made up of two α chains and two β chains; in SCD patients, a glutamate to valine amino acid substitution in the β chains causes abnormal hemoglobin S (HbS), which polymerizes in the deoxygenated form inside erythrocytes, resulting in altered (sickled) erythrocyte shape.[32-36] Hemoglobin C (HbC) is another abnormal hemoglobin in which glutamic acid residue at the sixth position of the β-chain is replaced with a lysine. In addition to the morphological changes, SCD leads to increased erythrocyte rigidity and adhesiveness.[35,37] The pathological HbS can bind to the membrane cytoskeleton made of spectrin and other proteins and modify its structure and mechanical properties.[38] Remarkably, altered mechanical properties of the erythrocyte membrane have been found in SCD patients with a single mutation in each of the two β subunits in HbS (HbSS) or HbC (HbCC) but also in patients with different mutations in each HbS and HbC subunit (HbSC).[23]

Here, we study differences in clot contraction and polyhedrocyte formation between healthy donors and SCD patients, while

probing erythrocyte rigidity as a mechanism for the observed decrease in blood clot contraction in SCD patients.

2 | METHODS

2.1 | Human subjects, blood sample collection and processing

Sixteen patients with SCD and 52 healthy subjects donated blood following informed consent in accordance with the protocols approved by the institutional review boards of the University of Pennsylvania (Philadelphia, Pennsylvania, USA) or Emory University (Atlanta, Georgia, USA). Adult subjects with SCD were selected for study based solely upon their transfusion history, absence of anticoagulants, and willingness to participate and, consequently, typify the characteristics of this patient population in general. One of 18 subjects was prescribed but thought to be noncompliant with hydroxyurea; no patients were offered L-glutamine as a therapeutic option. Subjects were not known to be taking aspirin or other anti-platelet agents on a regular basis at the time of blood collection. Other clinical characteristics of the SCD patients can be found in Table S1 in supporting information.

Blood samples were collected by venipuncture into 3.2% trisodium citrate 9:1 by volume, stored at room temperature and used within 4 h. Erythrocytes were isolated through centrifugation of citrated whole blood at 200 g for 10 min and were washed three times in phosphate-buffered saline (PBS), pH 7.4, by repeated centrifugation. The supernatant of the whole blood comprised platelet-rich plasma (PRP). To promote formation and polymerization of deoxyhemoglobin, in some experiments erythrocytes were deoxygenated by placing a whole blood sample into a 2-ml multi-well plate in an Eppendorf ThermoMixer with a custom designed mount that provides an air-tight seal for the inlet/outlet nozzles. Samples were flushed with argon for 30 min at 37°C under gentle agitation to maintain the maximal surface area for mixing.

2.2 | Scanning electron microscopy

Contracted clots were formed by addition of human thrombin (1 U/ml final concentration) and $CaCl_2$ (2 mM) into citrated native or reconstituted whole blood; activated samples were then transferred to Eppendorf tubes pre-coated with a residual layer of 4 vol% Triton X-100 in PBS to prevent fibrin sticking and incubated at 37°C for 30+ mins. Sediments of centrifuged erythrocytes or contracted blood clots were fixed in 2% glutaraldehyde in PBS, dehydrated in ascending concentrations of ethanol (30–100 vol%) and then dried with hexamethyldisilazane. The fixed blood clots were cut open to visualize the core (interior). Samples were sputter-coated with gold-palladium and imaged using an FEI Quanta 250 FEG scanning electron microscope. Erythrocytes were characterized as biconcave, biconcave-intermediate, polyhedral-intermediate, and polyhedral, as previously described.[11]

2.3 | Probing erythrocytes' membrane stiffness by atomic force microscopy

Microscope glass coverslips (22 × 22 × 0.16 mm, Fisher Scientific) were cleaned for 10 min using PDC-32G-2 Plasma Cleaner (Harrick Plasma). Afterward, a 20-µl drop of 0.1 mg/ml polyallylamine (Sigma-Aldrich) was placed on the glass, kept for 2 min, removed from the surface, and dried with a flow of air. To enforce erythrocyte attachment, a 20 µl drop of 0.1% glutaraldehyde (EMS) was placed on the glass slide pretreated with polyallylamine, kept for 2 min, and washed with 5 ml of milli-Q water. The remaining liquid was dried with a flow of air, making the surface ready for deposition of erythrocytes. Isolated erythrocytes were washed three times in Alsever's solution (Sigma-Aldrich) and stored at 8°C for up to 3 days. Prior to use, the erythrocytes were re-suspended in PBS and added to the modified glass coverslip. Erythrocytes were allowed to attach for 3 mins and then washed with PBS to remove the unattached cells. Residual washing buffer was removed from the surface gently without letting it dry completely. Cells were overlaid with 400 µl of PBS. Force-distance curves generated during controlled indentation of erythrocytes were obtained using an MFP-3D microscope (Asylum Research–Oxford Instruments) in a force mapping mode using long triangular cantilevers TR400PB (Olympus) with square pyramidal tips. The cantilever spring constant (about 0.02 nN/nm) was calibrated prior to each experiment by a thermal fluctuations method.[39,40] Cells were chosen visually using the embedded optical microscope. Not less than 10 different cells were measured for each blood sample. Each cell was characterized by 10–20 force-distance curves evenly distributed over the cell surface. We used an indentation force of 0.5 nN and an indentation rate of 2 µm/sec.

Indentation force-distance curves were analyzed using the modified Hertzian model developed by Sneddon for pyramidal indenters.[41] For a four-sided pyramidal indenter, the force as a function of indentation is described by the following equation:

$$F = \frac{3E\tan\theta}{4(1-\nu^2)}\delta^2,$$

where E is the apparent Young's modulus, ν is the Poisson ratio, δ is the indentation depth, and θ is the pyramid angle. The Poisson ratio was set at 0.5 to account for material incompressibility. An indentation range of 0–100 pN was analyzed. Each cell was characterized by its median Young's modulus, then the overall Young's modulus for the sample was obtained as the median of all measured cells.

2.4 | Kinetics of blood clot contraction

Blood was stabilized with citrate and clotting was initiated using 2 mM $CaCl_2$ and 1 U/ml thrombin (final concentrations). These conditions were previously optimized for rapid clot formation and prevention of erythrocyte settling.[16] Immediately following activation, samples were transferred to plastic cuvettes that were pre-lubricated

with 4 vol% Triton X-100 in PBS. The 12 mm × 7 mm × 1 mm cuvettes were then transferred into a thermostatic chamber (37°C) of the Thrombodynamics Analyser System. Changes in clot size were assessed every 15 s for 20 mins. The extent of clot contraction was determined relative to the initial clot size at t = 0 taken as 1. Lag time was determined as the time it took to reach 5% contraction. The mechanical work done by the contracting platelets is represented as the area under the curve. The phases of clot contraction were determined by fitting the kinetic curve with a piecewise function where the first phase is exponential, the second phase is linear, and the third phase is exponential, as previously described.[16]

2.5 | Statistical analysis

All statistics were completed using Prism GraphPad 6.0. All data is presented as mean ± standard error of the mean. Samples were analyzed for statistical significance using two-tailed unpaired *t*-tests with alpha = 0.05. Normality of distribution was determined with a D'Agostino and Pearson test. Significance is represented as $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

3 | RESULTS

3.1 | Clot contraction is delayed and reduced in SCD patients

Optical tracking of clot size in blood obtained from patients with SCD revealed significant modulation of all the parameters of clot contraction compared to healthy subjects (Figure 1). The average extent of clot contraction at 20 min was reduced by 45% (Figure 1A), the average lag time was prolonged 2.5-fold (Figure 1B), the average velocity was slower by 60% (Figure 1C), and area under the curve (work done by platelets during contraction) was smaller by about one half (Figure 1D). Correlation analysis of the clot contraction parameters revealed significant strong correlations between extent of contraction, lag time, average velocity, and area under the curve (Table S2 in supporting information), suggesting that there is a common reason for the reduced contraction of blood clots in SCD patients that affects various mechanisms and stages of contraction reflected by these parameters. There were no significant differences in extent of clot contraction in the blood of patients with HbSS or HbSC mutations in hemoglobin (Figure S1 in supporting information). Also, there was no difference in the extent of clot contraction between oxygenated and deoxygenated samples from SCD patients (Figure S1 in supporting information). We noted that there is little sickling of red blood cells (RBCs) due to the duration of deoxygenation. The last two results suggest that the solubility and/or predisposition to intracellular polymerization of deoxyhemoglobin are not responsible for the impaired blood clot contraction in SCD patients.

3.2 | Impaired phase kinetics of clot contraction in SCD patients

As we showed earlier, clot contraction occurs in three kinetically and mechanistically distinct phases, with Phase 1 corresponding to initiation of contraction (~100 s), Phase 2 to linear contraction (~250 s), and Phase 3 to mechanical stabilization.[16] Non-linear regression analysis was completed on the averaged kinetic curves of clot contraction in the blood of SCD patients versus healthy subjects (Figure 2). The quantitative results shown in Table 1 revealed that the patients had a 54% reduction in the averaged rate constant for Phase 2 and a 44% reduction in the rate constant associated with Phase 3 (Table 1), indicating impaired mechanical compaction of erythrocytes during shrinkage of clots formed in the blood of SCD patients. Visually, SCD patients have impaired formation of polyhedrocytes, more fibrin

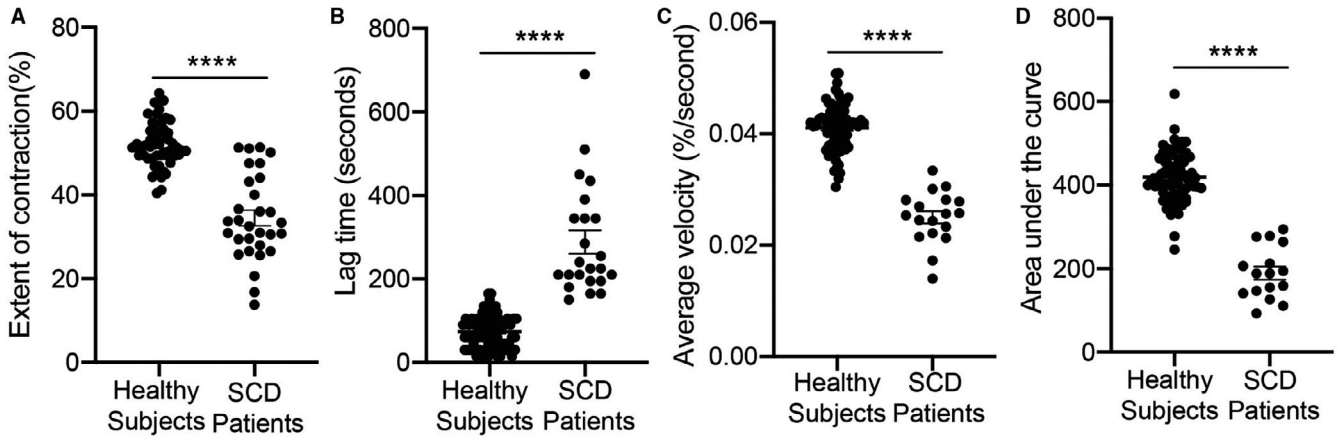


FIGURE 1 Parameters of blood clot contraction in sickle cell disease (SCD) patients and healthy individuals. Optical tracking of contracting blood clots was used to compare (A) extent of clot contraction at 20 min, (B) lag time, (C) average velocity, and (D) area under the kinetic curve. Parameters for healthy individuals ($n = 52$) and patients with SCD ($n = 16$) were compared using an unpaired, 2-tailed student *t*-test. $^{****}P < .0001$

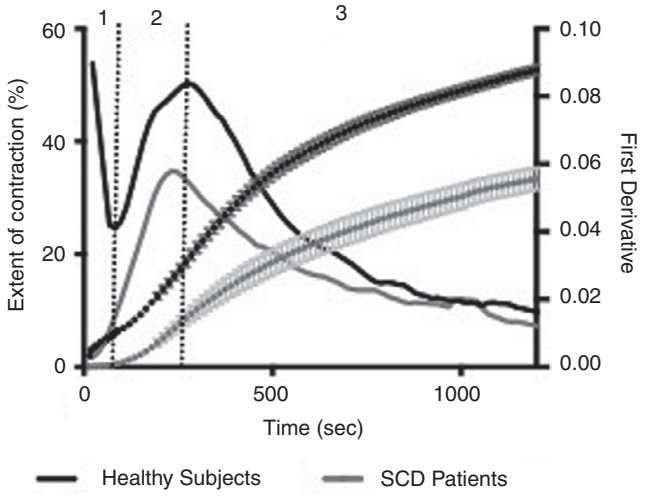


FIGURE 2 Averaged kinetic curves of clot contraction for sickle cell disease (SCD) patients (n = 16) and healthy subjects (n = 52). Optical tracking was used to measure the extent of clot contraction every 15 s over the course of 20 min. The instantaneous first derivative was used to define transitions between phases and calculate rate constants of each phase of contraction. Data points in the curves represent mean ± standard error of the mean from individual kinetic curves. Dashed vertical lines denote the transitions between phases

TABLE 1 Kinetics and phase analysis of clot contraction with clotting of the blood of sickle cell disease patients and healthy subjects

Rate constants	Healthy subjects (n = 52)	Sickle cell disease patients (n = 16)
Phase 1, 1/s	0.023 ± 0.005	Not detectable
Phase 2, %/s	0.072 ± 0.003	0.0305 ± 0.0033****
Phase 3, 1/s	0.0026 ± 0.0001	0.0011 ± 0.0001****

****P < .0001.

in the interior of the clot, and increased space within clots formed *in vitro* (Figure 3B,D) compared to healthy subjects (Figure 3A,C). This observation further supports the conclusion that clot contraction and mechanical compaction of erythrocytes is altered in SCD patients compared to healthy subjects. A priori, this alteration may occur as a result of increased stiffness of the clot due to heightened stiffness/rigidity of erythrocytes at the same hematocrit. This conclusion is reinforced as the hematocrit in most SCD patients is lower than in controls (Table S1 in supporting information).

3.3 | Increased erythrocyte rigidity in SCD patients

To test the latter possibility, using atomic force microscopy, we measured the elastic properties of erythrocytes isolated from the blood of SCD patients versus healthy subjects by controlled indentation of the plasma membrane at randomly selected points. The average Young's modulus of erythrocytes from SCD patients was 4-folder higher than erythrocytes from healthy individuals (Figure S2 in supporting information), directly confirming a substantial increase of the erythrocyte rigidity in SCD. To examine whether an increase in erythrocyte rigidity corresponded to the altered clot contraction and decreased polyhedrocyte formation observed in SCD patients, in the following experiments we modulated the stiffness of erythrocytes and evaluated the effect of these modifications on clot contraction in conjunction with erythrocyte deformability.

3.4 | Effects of chemically induced erythrocyte rigidity on polyhedrocyte formation

Because transformation of biconcave erythrocytes to polyhedrocytes during clot contraction has a purely mechanical nature,[1] we used centrifugation of erythrocytes with various rigidities to mimic

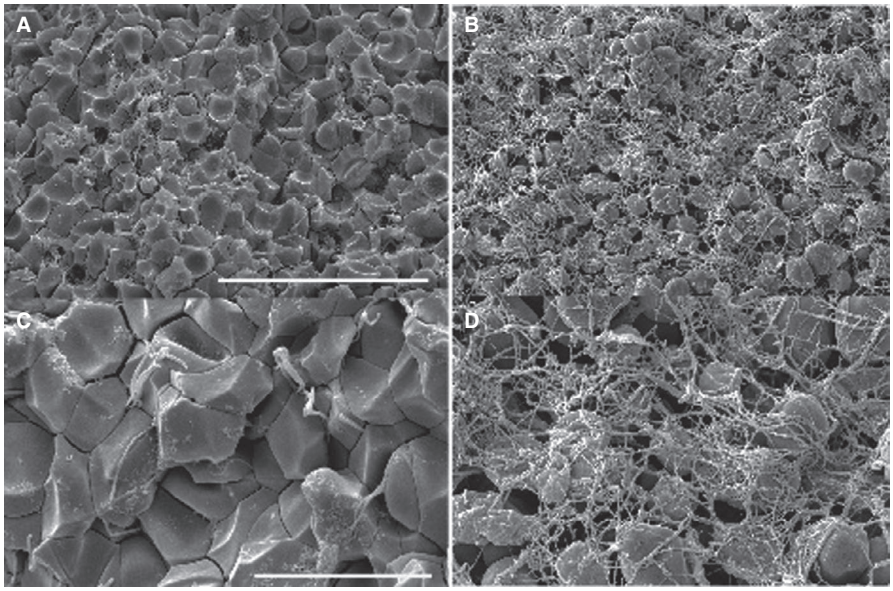


FIGURE 3 Representative scanning electron microscopy images of the core of contracted clots formed from the blood of (A,C) healthy subjects and (B,D) sickle cell disease (SCD) patients, showing the different degree of compaction of red blood cells and their transformation to polyhedrocytes, as well as absence (A, C) and presence (B, D) of fibrin in the interior of the blood clots. Scale bar for (A,B) is 30 μm and for (C,D) is 10 μm

the contractile forces generated by platelets and establish the mechanistic link between erythrocyte rigidity and their conversion to a polyhedral shape. By varying accelerations from 1 g (sedimentation due to gravity) to 7000 g, we were able to induce formation of a tessellated network of polyhedrocytes with unmodified normal human erythrocytes at accelerations as low as 1000 g (Figure 4). Centrifuged erythrocytes, unlike those naturally compressed during clot contraction, largely developed intermediate polyhedral forms due to the uniaxial application of forces compared to isotropic application of forces in contracting blood clots. To examine if increased rigidity influences the formation of polyhedrocytes, human isolated erythrocytes were incubated with 0.03 and 0.06% glutaraldehyde, which has been previously shown to alter erythrocyte rigidity by up to a 6-fold increase in stiffness without altering other properties of the erythrocytes.[42,43] Treatment with glutaraldyhyde prior to centrifugation resulted in a noticeable decrease in polyhedrocyte formation (Figures 4 and 5), even at centrifugal forces higher than 1000 g; ~90% of untreated erythrocytes were polyhedral-like or polyhedral at 1000 g compared to <15% of erythrocytes treated with either concentration of glutaraldehyde (Figure 5A-C).

3.5 | Effects of naturally increased erythrocyte rigidity on clot contraction

To further explore the relationship between erythrocyte rigidity and blood clot contraction, we performed experiments using the naturally stiffer form of erythrocytes called ovalocytes from a llama. Llama ovalocytes (Figure 6A) are more rigid than human erythrocytes due to the presence of 2.5 times the amount of the structural

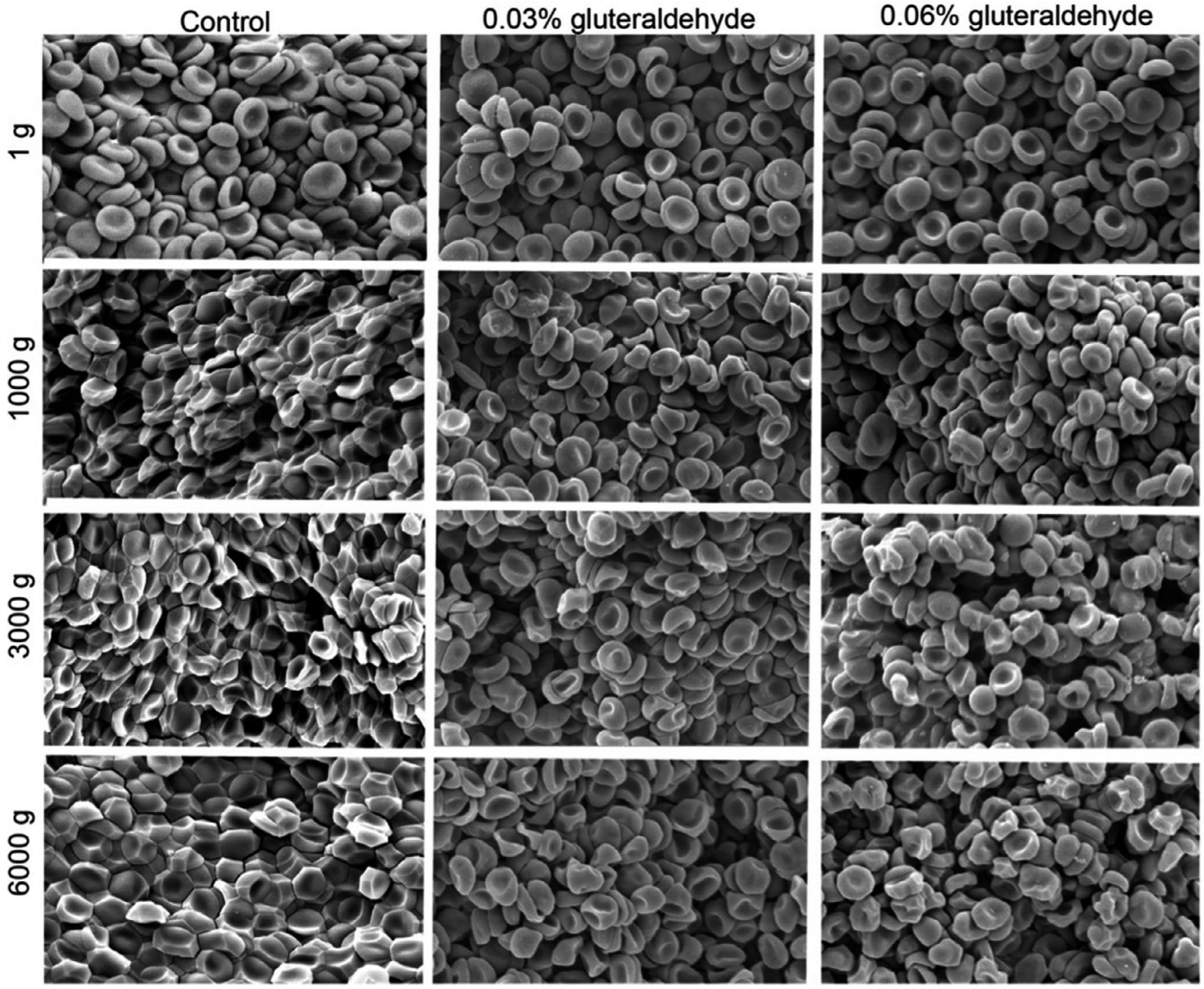


FIGURE 4 Representative scanning electron micrographs showing reduced polyhedral deformation in chemically rigidified erythrocytes. Centrifugation of normal untreated erythrocytes (control) revealed a transition from biconcave to polyhedral similar to what is observed in contracted blood clots. Polyhedral-intermediate forms are largely observed due to the anisotropic application of forces during centrifugation. This transition was mitigated in erythrocytes pretreated with 0.03% and 0.06% glutaraldehyde, which increased erythrocyte rigidity and reduced deformability (see Figure 5 for quantification)

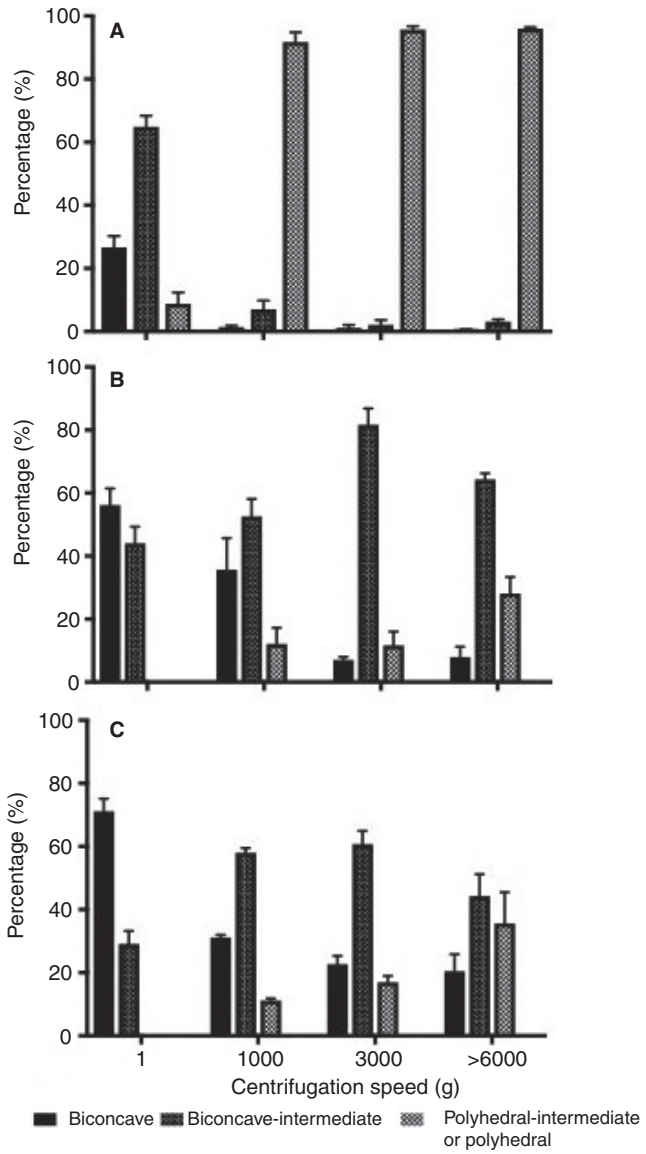


FIGURE 5 Quantification of polyhedrocyte formation in chemically rigidified erythrocytes. Erythrocytes were quantified for their shape transformation from biconcave to biconcave-intermediate, and polyhedral-intermediate or polyhedral following centrifugation. Deformations induced by centrifugation of untreated erythrocytes (A) and erythrocytes rigidified by incubation with (B) 0.03% or (C) 0.06% glutaraldehyde

protein spectrin,[44] so that they can resist larger osmotic pressures. Llama ovalocytes have the same length as human erythrocytes but their width is ~one half to one third (3 μm vs. 6.5–8.2 μm), leading to the llama ovalocytes having approximately one third the volume of human erythrocytes.[45] Isolated and washed llama ovalocytes or human erythrocytes were reconstituted at a volume fraction of 40% with human PRP at a platelet count of ~250 000/μl. In the presence of llama ovalocytes, there was a delay in the overall kinetics of clot contraction (Figure 6B, Figure S3 in supporting information), a 28% decrease in the extent of clot contraction (Figure 6C), and a reduction in the average velocity (Figure 6D). Specifically, there was a reduction in the rate of Phase 2 with the stiffer ovalocytes (Figure 6E). The addition of more platelets, or more contractile force, resulted in an extent of clot contraction closer to that of the human erythrocyte control, which shows that the increased contractile force can overcome the mechanical resistance of llama ovalocytes.

3.6 | Effects of biologically induced erythrocyte rigidity on clot contraction

As glutaraldehyde may have adverse effects on platelets, we utilized antibodies to the Wr^b epitope on erythrocytes, which target band 3/glycophorin a on the erythrocytes and have been shown to increase erythrocyte membrane rigidity by 1.4-fold.[42,46] Importantly, changes in band 3-cytoskeleton interactions have been linked to the increased rigidity of SCD erythrocytes.[47] The addition of 1.5 μM of Wr^b antibodies (~200,000 copies/cell) resulted in more rigid erythrocytes compared to healthy subjects (Figure 7A) and a statistically insignificant (due to high data scatter) but consistent trend toward a decrease in the parameters of clot contraction (Figure 7B, Figure S4 in supporting information). Assessment of erythrocytes from the core of clots showed that in the absence of Wr^b antibodies, 96% of RBCs were polyhedral-intermediate or polyhedral in shape, with 43% being polyhedral (Figure 7C,G) whereas, in the presence of Wr^b antibodies, only 17% of RBCs were polyhedral, and there were more partially compressed forms (Figure 7D,G). Centrifugation at accelerations greater than 1000 g resulted in the formation of polyhedral-intermediate RBCs in the absence of antibodies for 95% of RBCs (Figure 7E,H), while erythrocytes appeared less compacted in the presence of antibodies, with 47% of cells being biconcave or biconcave-intermediate (Figure 7F,H).

4 | DISCUSSION

Sickle cell disease affects 20–25 million people globally and is linked to an increased risk in thrombotic conditions, in particular venous thromboembolism.[48] Platelet-generated contractile forces are able to pack erythrocytes into the core of the clot and the cells are deformed into polyhedrocytes.[1] Here, we showed that clot contraction is delayed and reduced in SCD patients. Moreover, we used centrifugation to mimic the compressive forces generated by platelets and saw that in normal untreated erythrocytes, a tessellated network of polyhedrocytes formed at centrifugal forces of ~1000 g or higher (Figure 4). Treatment of erythrocytes with glutaraldehyde, which has been shown to decrease the deformability of the erythrocytes,[42,43] resulted in less erythrocyte deformation, not only at the threshold force of 1000 g but also at higher centrifugal forces. This confirms that erythrocyte rigidity may influence the compactness of the erythrocyte core of the contracted blood clot and further supports the idea that erythrocyte rigidity has the potential to influence the extent of clot contraction. In venous thrombi, erythrocytes comprise more than half the volume, making the role of erythrocyte rigidity critical for understanding the pathogenesis of venous thromboembolism in SCD patients.[49]

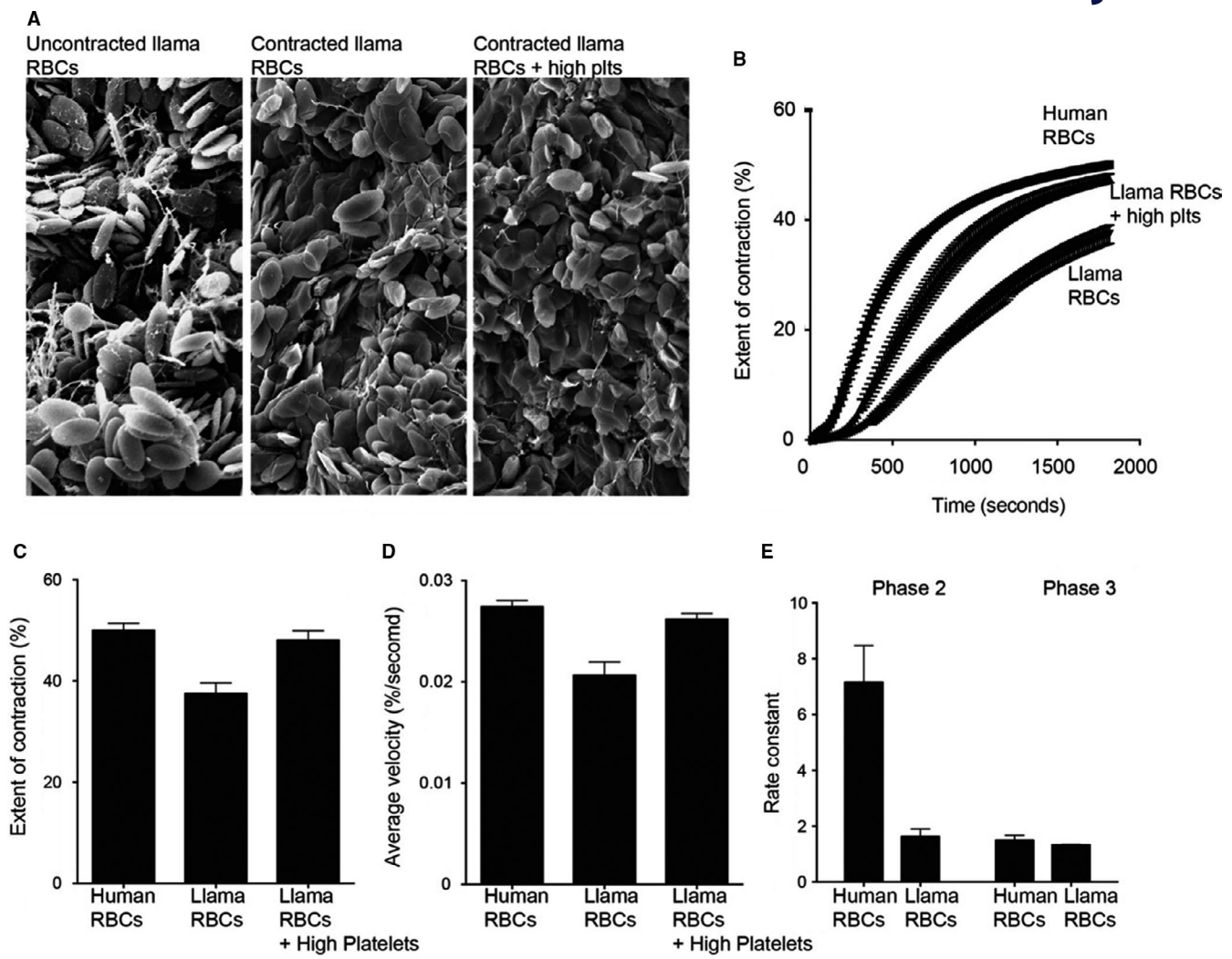
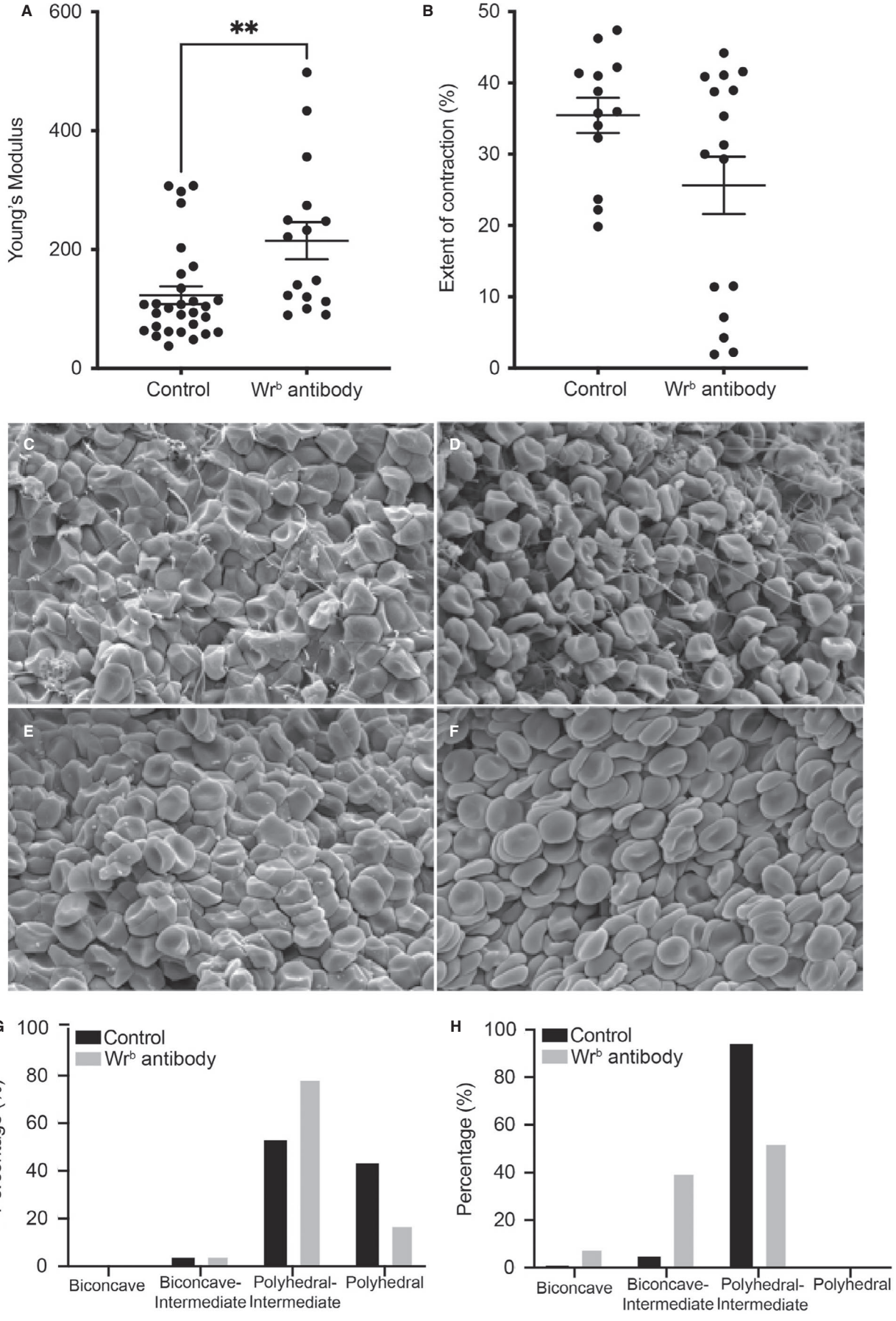


FIGURE 6 Clot contraction with naturally rigid llama ovalocytes. A, Representative scanning electron micrographs showing llama ovalocytes inside blood clots that were uncontracted, contracted with normal platelet count (~250,000/μl), or contracted using a higher platelet count (~400,000/μl). B, Kinetic curves of clot contraction in reconstituted blood to assess clot contraction in samples containing human erythrocytes or llama ovalocytes. C, Average extent of clot contraction at 20 mins, (D) average velocity, and (E) the rates of phases 2 and 3 were quantified in contracting reconstituted blood samples containing human or llama ovalocytes at various platelet counts

To assess the role of erythrocyte rigidity in modulating clot contraction, we performed experiments with naturally stiff ovalocytes from llamas added to platelet-rich human plasma. Llama ovalocytes are naturally more rigid than human erythrocytes because llamas need cells with an increased osmotic resistance that can withstand changes in osmotic pressure from ingestion of large volumes of water and subsequent dehydration. Llama ovalocytes displayed a decrease in the extent of clot contraction and reduction in the rate of Phase 2 of contraction similar to what was observed in the blood of SCD patients (Figure 5). The addition of more platelets, and consequently higher contractile forces, resulted in an increase in the extent of contraction and increased erythrocyte deformation compared to a lower platelet count. This further supports the idea that mechanical resistance conferred by the stiffer ovalocytes can be balanced by the generation of higher contractile forces. Likewise, the addition of rigidifying Wr^b antibodies to erythrocytes caused a decrease in polyhedrocyte formation and a trend toward a decrease in extent of clot contraction. Wr^b antibody–mediated increase in the stiffness of the erythrocytes is particularly important because the fact that a monovalent ligand induces rigidity shows that the mechanism is independent of membrane complex crosslinking and agglutination.

FIGURE 7 Contraction of blood clots formed in the presence of erythrocyte-rigidifying $Wright^b$ antibodies. Representative single donor experiment revealed that (A) Wr^b antibodies induced an increase in erythrocyte rigidity, which corresponded to (B) a decrease in the extent of contraction. Representative scanning electron microscopy showing erythrocyte deformation in the core of clots made (C) without or (D) with addition of Wr^b antibodies and following centrifugation of washed erythrocytes in the (E) absence or (F) presence of Wr^b antibodies. Distributions of red blood cell (RBC) shapes quantified from scanning electron microscopy images for (G) contracted clots and (H) centrifuged RBCs


A
600
400
200
0
Young's Modulus
**
Control
Wr^b antibody
B
50
40
30
20
10
0
Extent of contraction (%)
Control
Wr^b antibody
C
D
E
F
G
100
80
60
40
20
0
Percentage (%)
Control
Wr^b antibody
Biconcave
Biconcave-Intermediate
Polyhedral-Intermediate
Polyhedral
H
100
80
60
40
20
0
Percentage (%)
Control
Wr^b antibody
Biconcave
Biconcave-Intermediate
Polyhedral-Intermediate
Polyhedral

Pathological conditions that are associated with an increase in erythrocyte rigidity are associated with an increased risk for thrombotic conditions such as venous thromboembolism or ischemic stroke.[28] SCD is associated with platelet procoagulant properties,[50,51] activation of the coagulation cascade,[52,53] deformation of erythrocytes that become trapped in the microcirculation,[53] and impaired fibrinolysis.[25] Erythrocytes need to be highly deformable so that they can pass through capillaries for oxygen delivery,[54] but lower deformability of erythrocytes may also play a role in thrombosis.[55] We have previously shown that the extent of clot contraction can differentially influence the rate of fibrinolysis, where clots with impaired clot contraction have a reduced rate of internal fibrinolysis.[3] The reduction in clot contraction seen in SCD patients can also provide a potential mechanistic explanation for the impaired fibrinolysis described clinically in this patient population.

Clot contraction is differentially influenced by the molecular and cellular composition of the blood.[16] Platelet activation is necessary for clot contraction, so it is perhaps surprising that a procoagulant condition, such as SCD, would result in a reduced extent of clot contraction rather than enhanced platelet contractility. However, examination of clot contraction in SCD revealed a substantially reduced extent and rate of clot contraction in SCD patients compared to healthy individuals (Figure 1). These characteristics were observed in erythrocytes collected from a largely unselected group of patients with SCD who were not prescribed disease-specific therapeutics, demonstrating the ubiquity of this pathobiology in the sickle-cell population at large. We have previously shown that clot contraction is reduced in other patient populations such as ischemic stroke or venous thromboembolism, where the reduction in contraction was linked to platelet refractoriness or exhaustion.[4,5] Here we wanted to examine the contribution that the increased erythrocyte rigidity of SCD patients may have on the decreased extent of contraction. It is known that with increasing hematocrit, there is a decrease in the extent of contraction.[16] While SCD patients, both HbSS and HbSC, have a lower hematocrit than healthy individuals, they also have less deformable erythrocytes. It has been previously shown that erythrocytes from SCD patients have altered membrane rigidity, changes in their cytoskeleton, and increased viscosity[56-58]; these factors would change the bulk deformability of the cell and potentially make them less compressible.

Erythrocytes resist contraction through a compressive resistance to the active contractile forces.[15] The reduced rate of Phase 2 of clot contraction supports the hypothesis that the overall extent of contraction is reduced due to erythrocytes, as it is known that erythrocytes play a critical role during this phase of contraction.[16] However, there was no difference in extent of clot contraction following the deoxygenation of whole blood from SCD patients. It should be noted that there is little increase in sickling of RBCs, because of the relatively short time course of the deoxygenation. The lack of influence is likely also due in part to the increase in overall rigidity of HbS RBCs compared to healthy-subject RBCs even in the absence of deoxygenation (Figure S2).[59] SCD erythrocytes, even without deoxygenation and/or shape change, are on average eight times stiffer than healthy erythrocytes (Figure S2). The observed lack of difference comparing contraction between oxygenated and deoxygenated samples could also be influenced by an increase in the phosphatidylserine exposure on the erythrocyte surface,[60] which has been shown to contribute to a hypercoagulable state and enhance thrombin generation in other conditions,[61] and this may counteract any potential difference in extent of clot contraction due to erythrocyte rigidity.

In conclusion, we, for the first time, demonstrated the effect of pathologically increased erythrocyte rigidity on the extent of blood clot contraction and contraction-induced RBC deformation. Because clot contraction is associated with the formation of a good hemostatic seal and the restoration of blood flow past otherwise obstructive thrombi, our results reveal that conditions with more rigid erythrocytes, such as SCD, may increase the risk of obstructive thrombotic complications by impeding clot contraction. In addition, knowledge regarding erythrocyte rigidity and contraction of blood clots and thrombi can help to predict and evaluate the efficacy of therapeutic interventions, such as sensitivity to thrombolytics.

CONFLICTS OF INTEREST

The authors have no competing interests to disclose.

AUTHOR CONTRIBUTIONS

V.T., R.I.L., W.A.L, V.R.M., and J.W.W. designed experiments. V.T., A.P., C.N., C.V., E.W., O.A., and D.R.M. performed experiments. C.V. and D.L.S. contributed to antibody production, characterization, and modification. J.E.R. coordinated patient sample collection. D.L.S. provided llama blood. V.T., R.I.L., J.W.W., V.R.M., W.A.M., D.L.S., J.E.R., O.A., and D.L.M. interpreted the data. V.T., R.I.L., and J.W.W. contributed to the preparation of the manuscript. All authors edited the manuscript.

ORCID

Valerie Tutwiler https://orcid.org/0000-0003-1213-4447
Rustem I. Litvinov https://orcid.org/0000-0003-0643-1496
Wilbur A. Lam https://orcid.org/0000-0002-0325-7990
John W. Weisel https://orcid.org/0000-0002-9628-257X

TWITTER

Valerie Tutwiler @ ValTutwiler

REFERENCES

1. Cines DB, Lebedeva T, Nagaswami C, et al. Clot contraction: compression of erythrocytes into tightly packed polyhedra and redistribution of platelets and fibrin. *Blood*. 2014;123(10):1596-1603.
2. Muthard R, Diamond S. Blood clots are rapidly assembled hemodynamic sensors: flow arrest triggers intraluminal thrombus contraction. *Arterioscler Thromb Vasc Biol*. 2012;32(12):2938-2945.
3. Tutwiler V, Peshkova AD, Le Minh G, et al. Blood clot contraction differentially modulates internal and external fibrinolysis. *J Thromb Haemost*. 2019;17(2):361-370.
4. Peshkova AD, Malyasyov DV, Bredikhin RA, et al. Reduced contraction of blood clots in venous thromboembolism is a potential thrombogenicandembologenicmechanism.*THOpen*.2018;02(01):e104-e115.

5. Tutwiler V, Peshkova AD, Andrianova IA, Khasanova DR, Weisel JW, Litvinov RI. Contraction of blood clots is impaired in ischemic stroke. *Arterioscler Thromb Vasc Biol*. 2017;37(2):271-279.
6. Le Minh G, Peshkova AD, Andrianova IA, et al. Impaired contraction of blood clots as a novel prothrombotic mechanism in systemic lupus erythematosus. *Clin Sci*. 2018;132(2):243-254.
7. Peshkova AD, Safiullina SI, Evtugina NG, et al. Premorbid hemostasis in women with a history of pregnancy loss. *Thromb Haemost*. 2019;119(12):1994-2004.
8. Evtugina NG, Peshkova AD, Pichugin AA, Weisel JW, Litvinov RI. Impaired contraction of blood clots precedes and predicts postoperative venous thromboembolism. *Sci Rep*. 2020;10(1):18261.
9. Lam WA, Chaudhuri O, Crow A, et al. Mechanics and contraction dynamics of single platelets and implications for clot stiffening. *Nat Mater*. 2011;10(1):61-66.
10. Carr ME. Development of platelet contractile force as a research and clinical measure of platelet function. *Cell Biochem Biophys*. 2003;75(4):674-678.
11. Tutwiler V, Mukhitov AR, Peshkova AD, et al. Shape changes of erythrocytes during blood clot contraction and the structure of polyhedrocytes. *Sci Rep*. 2018;8(1):17907-17914.
12. Ząbczyk M, Sadowski M, Zalewski J, Undas A. Polyhedrocytes in intracoronary thrombi from patients with ST-elevation myocardial infarction. *Int J Cardiol*. 2015;179:186-187.
13. Leong L, Chernysh IN, Xu Y, et al. Clot stability as a determinant of effective factor VIII replacement in hemophilia A. *Res Pract Thromb Haemost*. 2017;1(2):231-241.
14. Litvinov RI, Khismatullin RR, Shakirova AZ, et al. Morphological signs of intravital contraction (retraction) of pulmonary thrombotic emboli. *BioNanoSci*. 2018;8(1):428-433.
15. Tutwiler V, Wang H, Litvinov RI, Weisel JW, Shenoy VB. Interplay of platelet contractility and viscoelasticity of fibrin/erythrocytes in blood clot contraction. *Biophys J*. 2017;112(4):714-723.
16. Tutwiler V, Litvinov RI, Lozhkin AP, et al. Kinetics and mechanics of clot contraction are governed by the molecular and cellular composition of the blood. *Blood*. 2016;127(1):149-159.
17. Rebsomen L, Tsimaratos M. Association of reduced red blood cell deformability and diabetic nephropathy. *Kidney Int*. 2005;67(5):2066.
18. Cicco G, Pirrelli A. Red blood cell (RBC) deformability, RBC aggregability and tissue oxygenation in hypertension. *Clin Hemorheol Microcirc*. 1999;21(3–4):169-177.
19. Agrawal R, Smart T, Nobre-Cardoso J, et al. Assessment of red blood cell deformability in type 2 diabetes mellitus and diabetic retinopathy by dual optical tweezers stretching technique. *Sci Rep*. 2016;6:15873.
20. Keymel S, Heiss C, Kleinbongard P, Kelm M, Lauer T. Impaired red blood cell deformability in patients with coronary artery disease and diabetes mellitus. *Horm Metab Res*. 2011;43(11):760-765.
21. Norton JW, Rand PW. Decreased deformability of erythrocytes from smokers. *Blood*. 1981;57:671-674.
22. Maciaszek JL, Lykotrafitis G. Sickle cell trait erythrocytes are significantly stiffer than normal. *J Biomech*. 2011;44(4):657-661.
23. Maciaszek JL, Andemariam B, Lykotrafitis G. Microelasticity of red blood cells in sickle cell disease. *J Strain Analysis*. 2011;46:368-379.
24. Huang Z, Hearne L, Irby CE, King SB, Ballas SK, Kim-Shapiro DB. Kinetics of increased deformability of deoxygenated sickle cells upon oxygenation. *Biophys J*. 2003;85(4):2374-2383.
25. Ataga KI, Orringer EP. Hypercoagulability in sickle cell disease: a curious paradox. *Am J Med*. 2003;115(9):721-728.
26. Ataga KI, Key NS. Hypercoagulability in sickle cell disease: new approaches to an old problem. *Blood*. 2007;2007(1):91-96.
27. Stein PD, Beemath A, Meyers FA, Skaf E, Olson RE. Deep venous thrombosis and pulmonary embolism in hospitalized patients with sickle cell disease. *Am J Med*. 2006;119(10):897.
28. Rahimi Z, Parsian A. Sickle cell disease and venous thromboembolism. *Mediterr J Hematol Infect Dis*. 2011;3(1):e2011024.
29. Switzer JA, Hess DC, Nichols FT, Adams RJ. Pathophysiology and treatment of stroke in sickle-cell disease: present and future. *Lancet Neurol*. 2006;5(6):501-512.
30. Faes C, Ilich A, Sotiaux A, et al. Red blood cells modulate structure and dynamics of venous clot formation in sickle cell disease. *Blood*. 2019;133(23):2529-2541.
31. Alberts B, Johnson A, Lewis J, Ralf M, Roberts K, Walter P. *Molecular biology of the cell*. New York, NY: Garland; 2002.
32. Steinberg MH, Brugnara C. Pathophysiological-based approaches to treatment of sickle cell disease. *Ann Rev Med*. 2003;54(1):89-112.
33. Ferrone FA. Polymerization and sickle cell disease: a molecular view. *Microcirculation*. 2004;11(2):115-128.
34. Noguchi CT, Schechter AN. Sickle hemoglobin polymerization in solution and in cells. *Ann Rev Biophys Biophys Chem*. 1985;14:239-263.
35. Turner MS, Wang J, Jones CW, Ferrone FA, Josephs R, Briehl RW. Fluctuations in self-assembled sickle hemoglobin fibers. *Langmuir*. 2002;18(19):7182-7187.
36. Christoph GW, Hofrichter J, Eaton WA. Understanding the shape of sickled red cells. *Biophys J*. 2005;88(2):7182-7187.
37. Aprelev A, Rotter MA, Etzion MA, Bookchin RM, Briehl RW, Ferrone FA. The effects of erythrocyte membranes on the nucleation of sickle hemoglobin. *Biophys J*. 2005;88(4):2815-2822.
38. Mohandas N, Evans E. Mechanical-properties of the red-cell membrane in relation to molecular-structure and genetic-defects. *Ann Rev Biophy Biomed*. 1994;23:787-818.
39. Saulson PR. Thermal noise in mechanical experiments. *Phys Rev D*. 1990;42(8):2437-2445.
40. Roters A, Johannsmann D. Distance-dependent noise measurements in scanning force microscopy. *J Phys Condens Matter*. 1996;8(41):7561-7577.
41. Sneddon IN. The relation between load and penetration in the axisymmetric boussinseq problem for a punch of arbitrary profile. *Int J Eng Sci*. 1965;3(638):47-57.
42. Pasvol J, Chasis JA, Mohandas N, Anstee DJ, Tanner MJA, Merry AH. Inhibition of malarial parasite invasion by monoclonal antibodies against glycophorin A correlates with reduction in red cell membrane deformability. *Blood*. 1989;74(5):1836-1843.
43. Mohandas N, Lie-Injo LE, Friedman M, Mak JW. Rigid membranes of malayan ovalocytes: a likely genetic barrier against malaria. *Blood*. 1984;63(6):1385-1392.
44. Smith JE, Mohandas N, Clark MR, Greenquist AC, Shohet SB. Deformability and spectrin properties in three types of elongated red cells. *Am J Hematol*. 1980;8(1):1-13.
45. Reynafarje C, Faura J, Villavicencio D, et al. Oxygen transport of hemoglobin in high-altitude animals (camelidae). *J Appl Physiol*. 1975;38(5):806-810.
46. Villa CH, Pan DC, Johnston IH, et al. Biocompatible coupling of therapeutic fusion proteins to human erythrocytes. *Blood advances*. 2018;2(3):165-176.
47. Xu Z, Zheng Y, Wang X, et al. Stiffening of sickle cell trait red blood cells under simulated strenuous exercise conditions. *Microsyst Nanoeng*. 2016;2(1):16061.
48. Noubiap JJ, Temgoua MN, Tankeu R, Tochie JN, Wonkam A, Bigna JJ. Sickle cell disease, sickle trait and the risk for venous thromboembolism: a systematic review and meta-analysis. *Thromb J*. 2018;16(1):27.
49. Chernysh IN, Nagaswami C, Kosolapova S, et al. The distinctive structure and composition of arterial and venous thrombi and pulmonary emboli. *Sci Rep*. 2020;10(1):5112.
50. Kenny MW, George AJ, Stuart J. Platelet hyperactivity in sickle-cell disease: a consequence of hyposplenism. *J Clin Pathol*. 1980;33(7):622-625.
51. Famodu AA, Oduwa D. Platelet count and platelet factor 3 (PF-3) availability in sickle cell disease. *Br J Biomed Sci*. 1995;52(4):323-324.

52. Westerman M, Pizzey A, Hirschman J, et al. Microvesicles in haemoglobinopathies offer insights into mechanism of hypercoagulability, haemolysis and the effect of therapy. *Br J Haematol*. 2008;142(1):126-135.
53. Bunn HF. Pathogenesis and treatment of sickle cell disease. *N Engl J Med*. 1997;337(11):762-769.
54. Huisjes R, Bogdanova A, van Solinge WW, Schiffelers RM, Kaestner L, van Wijk R. Squeezing for life – properties of red blood cell deformability. *Front Physiol*. 2018;9:656.
55. Litvinov RI, Weisel JW. Role of red blood cells in hemostasis and thrombosis. *ISBT Science Series*. 2017;112(1):176-183.
56. Kaul DK, Fabry ME, Windisch P, Baez S, Nagel RL. Erythrocytes in sickle cell anemia are heterogeneous in their rheological and hemodynamic characteristics. *J Clin Investig*. 1983;72(1):22-31.
57. George A, Pushkaran S, Li L, et al. Altered phosphorylation of cytoskeleton proteins in sickle red blood cells: the role of protein kinase C, rac GTPases, and reactive oxygen species. *Blood Cells Mol Dis*. 2010;45(1):41-45.
58. Byun H, Hillman TR, Higgins JM, et al. Optical measurement of biomechanical properties of individual erythrocytes from a sickle cell patient. *Acta Biomater*. 2012;8(11):4130-4138.
59. Gutierrez M, Shamoun M, Tanski T, Eniola-Adefeso L. Characterization of bulk rigidity of rigid red blood cell populations in sickle cell patients using a parameterization model of ektacytometry. *Blood*. 2019;134(Supplement_1):3572.
60. Weiss E, Rees DC, Gibson JS. Role of calcium in phosphatidylserine externalisation in red blood cells from sickle cell patients. *Anemia*. 2011;2011:1-8.
61. Bonomini M, Sirolli V, Merciaro G, et al. Red blood cells may contribute to hypercoagulability in uraemia via enhanced surface exposure of phosphatidylserine. *Nephrol Dial Transplant*. 2005;20(2):361-366.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Tutwiler V, Litvinov RI, Protopopova A, et al. Pathologically stiff erythrocytes impede contraction of blood clots. *J Thromb Haemost*. 2021;19:1990–2001. https://doi.org/10.1111/jth.15407