Preclinical evaluation of the preservation of red blood cell concentrates by hypoxic storage technology for transfusion in sickle cell disease

Sickle cell disease (SCD) is a congenital hemolytic anemia characterized by vaso-occlusive crises. Red cell exchange transfusion is commonly used to treat both acute and chronic complications.[1,2] Improving the quality of stored red blood cells (RBC) is essential to reduce transfusion frequency and to limit their pathological interactions with hemolysis damaged endothelial cells (EC).[3,4] Several groups demonstrated an improvement of RBC properties when stored under hypoxia,[5–9] mainly due to a reduction of oxidative damage. However, the use of hypoxic red blood cell concentrates (RBCC) for transfusion of SCD patients has not been specifically explored yet. To that purpose, we evaluated hypoxic RBC properties that could be relevant for transfusion of SCD patients throughout storage (up to 42 days at 4°C) compared to conventionally-stored normoxic RBCC. We demonstrated a non-inferiority of hypoxic storage on i) RBC senescence parameters (intracellular calcium entry, reactive oxygen species [ROS] and phosphatidylserine exposure [PS]), ii) activation and interaction of EC with sickle RBC and an improvement in i) hemolysis, ii) RBC adhesion to thrombospondin 1 (TSP-1), compared to normoxic storage. These results suggest that RBCC hypoxic storage could meet the safety criteria for transfusion of SCD patients and may provide clinical benefits, specifically by reducing free hemoglobin generation through storage (which could damage EC during transfusion) and RBC interaction with TSP-1 (involved in acute chest syndrome [ACS][10] and RBC recruitment on EC in SCD[11]).

For each experiment, two whole blood units were collected from two compatible ABO healthy volunteers, leukofiltred, pooled together and split into two RBCC, one of them being deoxygenated. Both hypoxic and normoxic bags were stored for 42 days at 4°C (Figure 1A). RBCC were successfully deoxygenated with an oxygen saturation remaining inferior to 21% throughout storage (*Online Supplementary Table S1*). Hemolysis was measured in RBCC supernatants by the quantification of free oxyhemoglobin (HbO_2) (Figure 1B). Despite a higher HbO_2 concentration at the beginning of the storage in the hypoxic bags, accumulation of free HbO_2 over time was significantly reduced under hypoxia. However, this free hemoglobin (Hb) was more oxidized under hypoxia, as suggested by the free methemoglobin (MetHb) concentration (*Online Supplementary Figure S1A*), while intra-RBC MetHb showed no significant difference between both storage conditions (*Online Supplementary Figure S1B*).

RBC senescence was evaluated by the measurement of intracellular ROS, calcium influx into RBC and extracellular membrane exposure of phosphatidylserine (PS). While no difference in ROS concentrations was found on day 0, a significant reduction (16%) was observed in RBC stored under hypoxia for 21 days (Figure 1C, when both groups were compared at one time point). Calcium influx (Figure 1D) was significantly decreased in hypoxic RBC on day 21 and 42 (reduction of 12% and 18%, respectively, when both groups were compared at one time point). PS exposure at the RBC surface was similar between both storage methods (*Online Supplementary Figure S1C*). Evaluation of intracellular ROS and calcium influx over time during the entire storage period only showed a trend in favor of hypoxic storage, probably due to the small sample size.

Increased adhesion to TSP-1 is one of the senescence features that appears throughout RBC storage. RBC adhesion to TSP-1 was measured under physiologic flow with a shear stress of 0.5 dyn/cm^2, on RBC incubated for 24 hours (h) in plasma from healthy donors (HD) or SCD patients at steady state or during an ACS (Figure 2). At the beginning of the storage (day 0, Figure 2A) hypoxia reduced RBC adhesion to TSP-1, irrespective of plasma origin. Evaluation of adhesion over time during the entire study period showed a significant increase with storage time and hypoxic conditions consistently trended lower than normoxic but the differences were not significant (Figure 2B to D), probably due to the low sample size. In order to assess RBC binding force to TSP-1, shear stress was increased to 2, then 5 dyn/cm^2 after the initial adhesion step at 0.5 dyn/cm^2 (*Online Supplementary Figure S2* and data not shown for 5 dyn/cm^2). Similar results were obtained with no significant difference between groups on day 21 and 42 of storage. At 5 dyn/cm^2 hypoxic RBC incubated with HD plasma showed a significantly reduced adhesion over time (Figure 2E). No significant difference was observed between non-incubated hypoxic and normoxic RBC (*Online Supplementary Figure S2E*).

In order to evaluate the effect of hypoxic RBC perfusion on EC, human umbilical vein endothelial cells (HUVEC) were perfused for 4 h with hypoxic or normoxic RBC (sampled on day 0, 21, 42) diluted in HD serum and supplemented with or without 10% hemolysate (conditioning step) to reproduce acute hemolysis that can be observed in SCD patients (Figure 3A). HUVEC activation marker levels (E-selectin and Intercellular Adhesion Molecule 1) were assessed by flow cytometry and showed no signifi-

cant differences between hypoxic and normoxic RBC perfusion (data not shown). Adhesion flow experiments with SCD blood were also performed after HUVEC conditioning, in order to evaluate the effect of hypoxic RBC perfusion on interactions between sickle RBC and EC (Figure 3B and C). No significant difference was observed between normoxic and hypoxic RBC perfusions except a significant 20% decrease of RBC adhesion on EC previously perfused

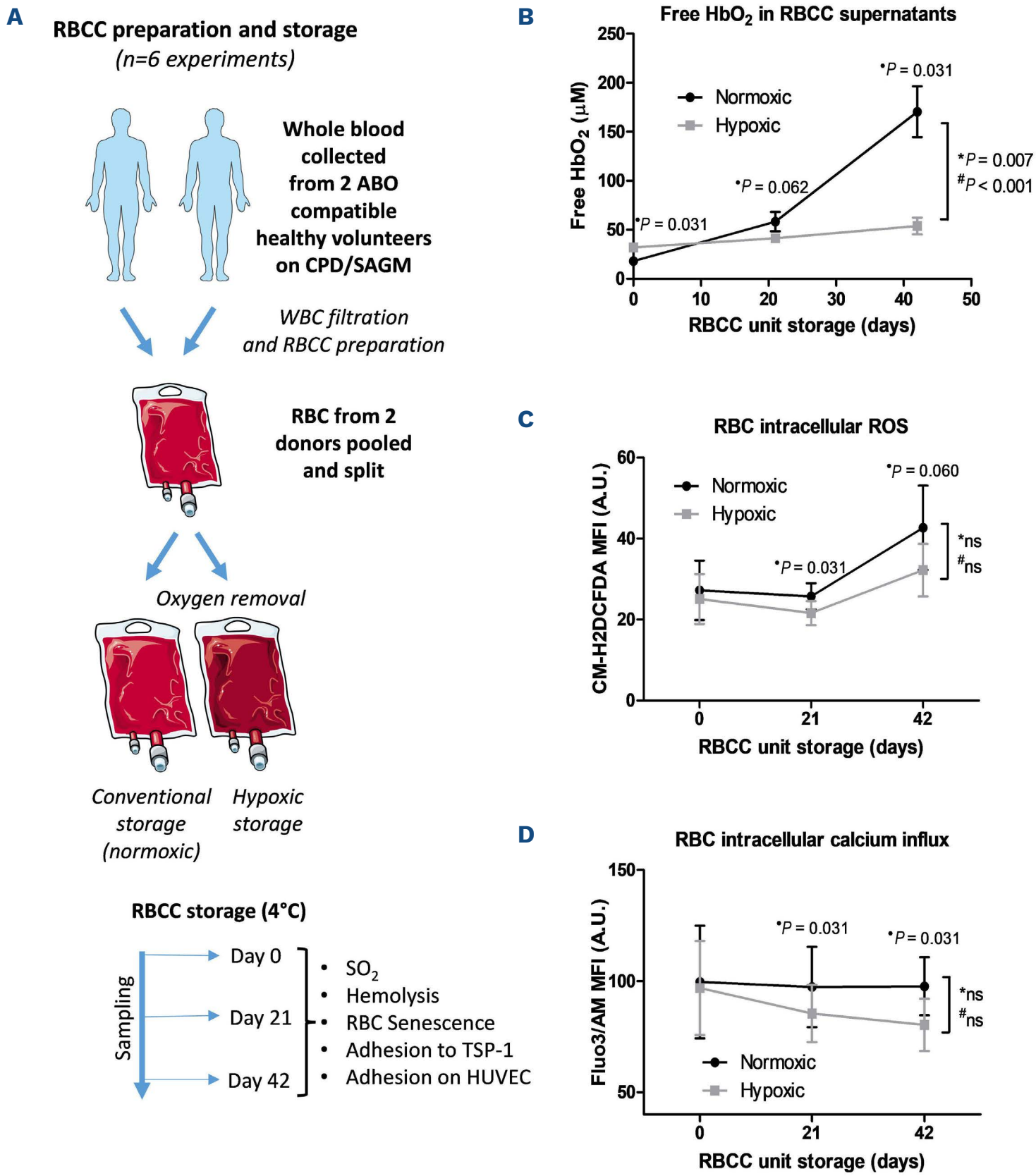

Figure 1. Hemolysis and senescence measurements during red blood cell concentrates storage. (A) Diagram of blood bag preparation and storage. Whole blood was collected from two ABO compatible healthy donors for each experiment (n=6). After white blood cell (WBC) removal, red blood cells (RBC) were pooled and split into two red blood cell concentrate (RBCC) bags. One of them was conventionally stored (normoxic) while the other underwent deoxygenation through Hemanext Inc. technology. Both bags were stored at 4°C for 42 days. Sampling was performed on day 0, 21 and 42 of storage for blood gas, hemolysis, senescence and adhesion measurements. (B) Free oxyhemoglobin (HbO_2) measured by spectrophotometry in normoxic (black) or hypoxic (grey) bag supernatants throughout the 42 days of storage. Mean +/- standard error of the mean (SEM) of 6 independent experiments. *Storage method effect, #interaction time/storage method, •comparison between hypoxic and normoxic storage at one time point. (C and D) Senescence parameters measured by flow cytometry in RBC from normoxic (black) or hypoxic (grey) bags throughout 42 days of storage, (C) intracellular reactive oxygen species (ROS) concentration measured using CM-H2DCFDA fluorescent probe, (D) calcium influx measured using Fluo-3/AM fluorescent probe. Mean +/- SEM of 6 independent experiments. *Storage method effect, #interaction time/storage method, •comparison between hypoxic and normoxic storage at one time point. ns: not significant. MFI: mean fluorescence intensity, A.U.: arbitrary units.

with hemolysate and hypoxic RBC collected at day 42, compared to normoxic RBC (Figure 3B).

Altogether these data suggest that hypoxic RBC exposure under flow does not excessively damage EC compared to perfusion with normoxic RBC and could even be beneficial in specific contexts (at the end of storage and in the presence of acute hemolysis).

Hypoxic storage of RBCC was demonstrated to reduce

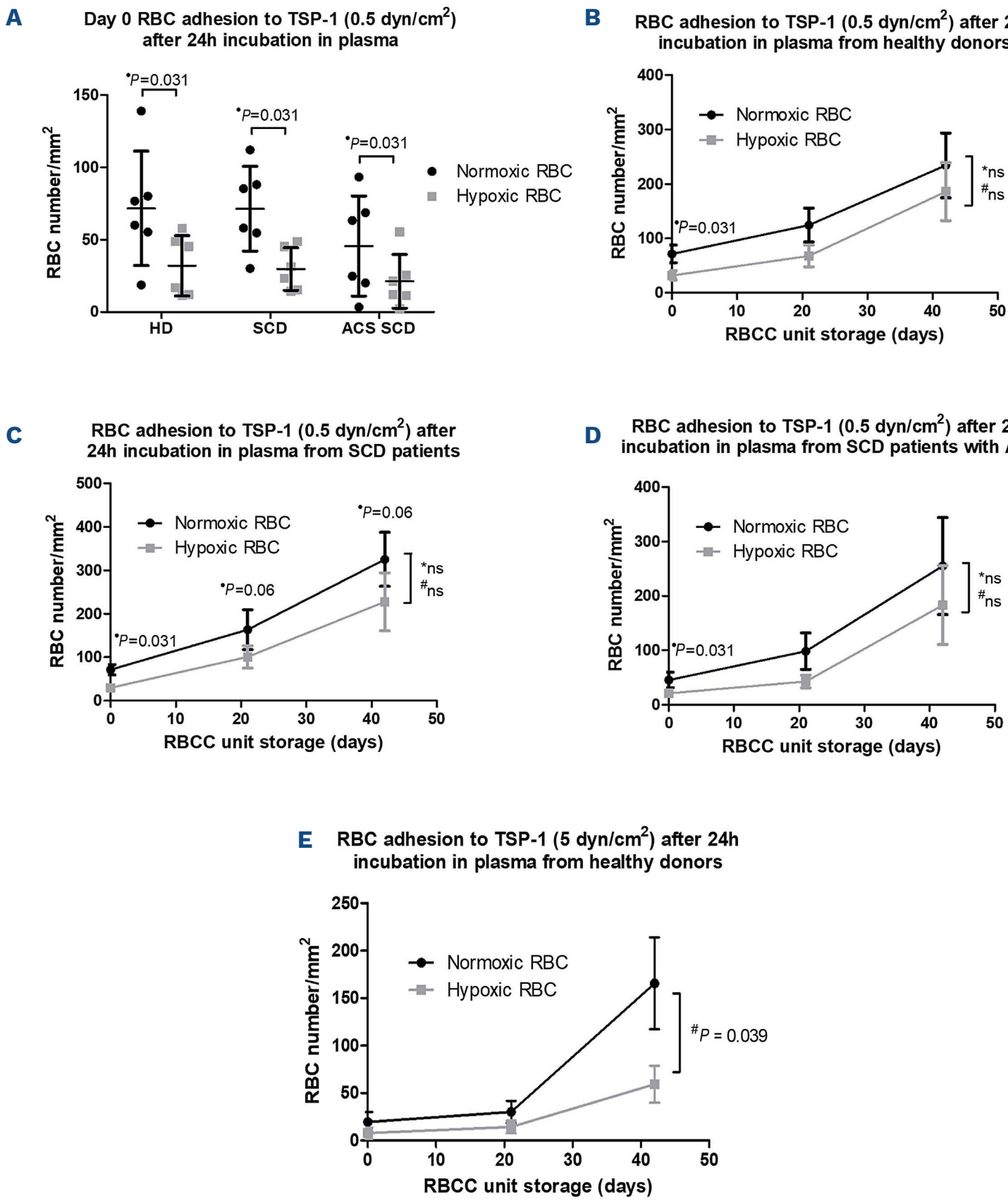

Figure 2. Red blood cell adhesion to TSP-1 at 0.5 and 5 dyn/cm². Red blood cells (RBC) from normoxic or hypoxic bags were sampled on day 0, 21 and 42 of storage and incubated at 37°C for 24 hours (h) with plasma from healthy donors (HD), steady state sickle cell disease patients (SCD) or sickle cell disease patients with an acute chest syndrome (ACS SCD). RBC were washed and resuspended at 0.66% hematocrit and perfused into TSP-1 coated channels at a shear stress of 0.5 dyn/cm² for 5 minutes (min) for adhesion, then washed for 5 min before quantification of adherent RBC on a light microscope. RBC adhesion strength was evaluated by increasing shear stress to 2 dyn/cm² for 2 min 30 seconds (sec) and at 5 dyn/cm² for 2 min 30 sec (adherent RBC quantification at the end of each period). (A) RBC adhesion to TSP-1 (0.5 dyn/cm²) on day 0 of storage after 24 h incubation with HD, SCD or ACS SCD plasma. RBC adhesion to TSP-1 (0.5 dyn/cm²) on day 0, 21 and 42 of storage after 24 h incubation with (B) HD plasma , (C) SCD plasma, (D) ACS SCD plasma or (E) HD plasma at 5 dyn/cm². Mean +/- standard error of the mean of 6 independent experiments, *storage method effect, #interaction time/storage method, •comparison between hypoxic and normoxic storage at one time point. ns: not significant. RBCC: red blood cell concentrate.

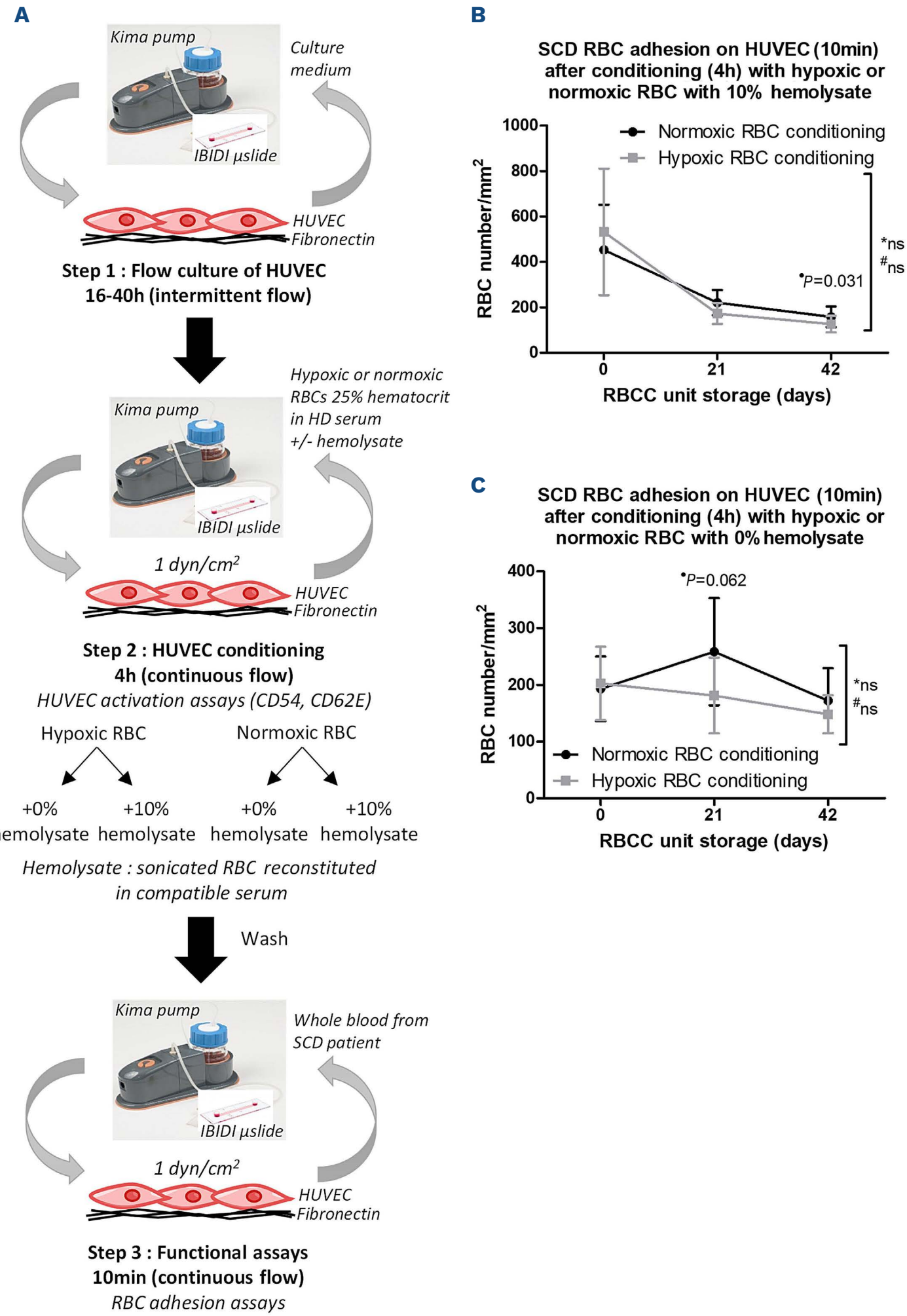

Figure 3. Red blood cell adhesion to endothelial cells. (A) Microfluidic model diagram. Human umbilical endothelial cells (HUVEC) were perfused for 16-40 hours (h) with culture medium in microslides coated with fibronectin (step 1), then perfused for 4 h at 1 dyn/cm^2 with red blood cells (RBC) from normoxic or hypoxic bags, diluted at 25% in compatible serum (from healthy donor, HD) and with the addition (or not) of 10% hemolysate (step 2). After the conditioning step (step 2), HUVEC were washed with culture medium and perfused with whole blood from sickle cell disease (SCD) patients at 1 dyn/cm^2 for 10 minutes before a second wash with culture medium to assess RBC adhesion to endothelial cells. All steps (except washes) were performed at 37°C. (B and C) Adhesion of RBC from steady state SCD patients to HUVEC perfused for 4 h with normoxic or hypoxic RBC sampled on day 0, 21 and 42 of storage, diluted at 25% in HD serum and with (B) 10% hemolysate or (C) 0% hemolysate. Adherent RBC were quantified on a light microscope. Mean +/- standard error of the mean of 6 independent experiments, *storage method effect. #interaction time/storage method. •Comparison between hypoxic and normoxic storage at one time point. ns: not significant.

storage lesion and improve RBC quality *in vitro*,[5,6,8,9] as well as RBC survival after transfusion in healthy volunteers.[7,12] However, the use of hypoxic RBC for the transfusion of SCD patients had not yet been explored. In this study, we demonstrated a non-inferiority of RBCC hypoxic storage compared to standard normoxic RBCC conservation. Moreover, several parameters were shown to be improved with hypoxic storage, which suggests a potential beneficial impact for transfusion of SCD patients.

In accordance with previous studies performed with different hemolysis measurement methods,[7,9] we observed a reduced hemolysis in hypoxic bags over time, except at the beginning of storage. The observed increase in hemolysis on day 0 may be due to the extra preparation steps required for deoxygenation. However, although a hypoxic environment should reduce hemoglobin oxidation to methemoglobin, we still observed a higher methemoglobin concentration in the supernatant under hypoxia. This result is probably linked to the increased Hb autoxidation rate at low O_2 pressure.[13] However, the relatively low quantity of free Methemoglobin (23 mM on day 42 *vs.* 15 mM in normoxic supernatant), once diluted in patient circulation, does not seem to be a concern. In addition, we did not observe any difference in intra-RBC methemoglobin content.

We also evaluated the effect of hypoxia on RBC senescence markers, but the improvement observed was limited. Indeed, senescence marker appearance is also modest with conventional normoxic storage, which is consistent with previous studies and could have limited hypoxic storage impact. However, these improvements in the measured *in vitro* metrics of hypoxic RBC still support the improved post-transfusion recovery observed in healthy volunteers and could be of interest in reducing the frequency of transfusion in SCD patients.

This study also showed a significant decrease in the adhesion of hypoxic RBC to TSP-1, but only at the beginning of the storage. Such a result suggests that the reduced RBC adhesion to TSP-1 is not linked to a reduction of oxidative damage but rather to other mechanisms induced by hypoxia such as metabolic changes for example. However, a decreased interaction with TSP-1 may be beneficial to patients undergoing ACS, who present high TSP-1 plasma level.

Finally, this study did not identify any detrimental impact of hypoxic RBC on EC, only a slight reduction of their adhesion properties induced by hemolysate, towards RBC from SCD patients.

Hypoxic RBC were successfully transfused in healthy volunteers and were shown to have higher 24 h post-transfusion recovery after 42 days of storage[7,12]. However, SCD patients present specific features which could require an improved monitoring during clinical trials.

The first issue is the risk of oxygen capture by transfused RBC, at the expense of recipient's RBC, which could entail their sickling. In this respect, *in vitro* experiments were performed with a mixture of hypoxic RBC and RBC from SCD patients, which did not show any significant sickling increase.[14]

The second issue is the risk of pulmonary arterial vasoconstriction during transfusion. As a matter of fact, the transfusion of large amounts of hypoxic RBC could reduce vascular pO_2, which could lead to vasoconstriction. However, if donor RBC are infused slowly enough into the body, a sudden drop in vascular pO_2 may not be likely. Particular attention should be paid to patients with pulmonary hypertension during clinical trials since they could be more prone to vascular constriction.

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Disclosures

Hemanext Inc supplied the funding for hiring LB. AD and SSC are employees of Hemanext Inc. PB declares being member on a standing advisory council or committee consultancy for Addmedica, Roche, Bluebird bio, Emmaus, Agios, Global Blood Therapeutics, Novartis and Hemanext Inc. PB declares being co-founder of INNOVHEM. The other authors declare no competing financial interests.

Contributions

LB, PC, LK, GB, AJ, SA, NH and MS performed research. KAN, FP and PB designed the study. LB, LK, EA, and PB performed data analysis.

SP and KT selected and enrolled patients. TB and BM selected healthy donor samples and prepared red blood cell concentrates. LB, SSC and PB wrote the manuscript. FP, SSC, AD and PB supervised the study.

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Data-sharing statement
The data that support the findings of this study are available on request from the corresponding author, PB.

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