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Original article

Glucose 6-phosphate dehydrogenase deficient subjects may be better "storers" than donors of red blood cells

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ABSTRACT

Storage of packed red blood cells (RBCs) is associated with progressive accumulation of lesions, mostly triggered by energy and oxidative stresses, which potentially compromise the effectiveness of the transfusion therapy. Concerns arise as to whether glucose 6-phosphate dehydrogenase deficient subjects ($G6PD^-$), ~5% of the population in the Mediterranean area, should be accepted as routine donors in the light of the increased oxidative stress their RBCs suffer from. To address this question, we first performed morphology (scanning electron microscopy), physiology and omics (proteomics and metabolomics) analyses on stored RBCs from healthy or $G6PD^-$ donors. We then used an *in vitro* model of transfusion to simulate transfusion outcomes involving $G6PD^-$ donors or recipients, by reconstituting $G6PD^-$ stored or fresh blood with fresh or stored blood from healthy volunteers, respectively, at body temperature. We found that $G6PD^-$ cells store well in relation to energy, calcium and morphology related parameters, though at the expenses of a compromised anti-oxidant system. Additional stimuli, mimicking post-transfusion conditions (37 °C, reconstitution with fresh healthy blood, incubation with oxidants) promoted hemolysis and oxidative lesions in stored $G6PD^-$ cells in comparison to controls. On the other hand, stored healthy RBC units showed better oxidative parameters and lower removal signaling when reconstituted with $G6PD^-$ fresh blood compared to control. Although the measured parameters of stored RBCs from the G6PD deficient donors appeared to be acceptable, the results from the *in vitro* model of transfusion suggest that $G6PD^-$ RBCs could be more susceptible to hemolysis and oxidative stresses post-transfusion. On the other hand, their chronic exposure to oxidative stress might make them good recipients, as they better tolerate exposure to oxidatively damaged long stored healthy RBCs.

Abbreviations: AnnV, Annexin V; ATP, adenosine triphosphate; CPD, citrate-phosphate-dextrose; DPG, 2,3-diphosphoglycerate; Hct, hematocrit; Hb, hemoglobin; H_2O_2, hydrogen peroxide; G6PD, glucose-6-phosphate dehydrogenase; $G6PD^-$, G6PD deficiency; GSH, reduced glutathione; GSSG, oxidized glutathione; K^+, potassium; LDH, lactate dehydrogenase; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MetHb, methemoglobin; MPs, microparticles, microvesicles; NAD^+, oxidized form of nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NS, non-stored; PPP, pentose phosphate pathway; PS, phosphatidylserine; RBC, red blood cell; RFU, relative fluorescence units; ROS, reactive oxygen species; SAGM, saline-adenine-glucose-mannitol; tBHP, *tert*-Butyl hydroperoxide; UHPLC-MS, ultimate high pressure liquid chromatography-mass spectrometry

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1. Introduction

The pentose phosphate pathway (PPP) is the major generator of anti-oxidant reducing equivalents (reduced nicotinamide adenine dinucleotide phosphate, NADPH) in red blood cells (RBC). NADPH is generated by two reactions catalysed by the rate-limiting enzymes of the PPP, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase. NADPH and reduced glutathione (GSH) play a vital role in antioxidant defence by reacting with hydrogen peroxide (H_2O_2) and organic peroxides, as well as by maintaining thiol groups of hemoglobin (Hb) and other proteins and enzymes in the reduced state [1]. H_2O_2 can be detoxified by catalase which is also stabilized by tightly bound NADPH [2]. NADPH-methemoglobin (MetHb) reductase may use NAPDH to reduce the Fe^{3+} of MetHb to Fe^{2+}, thereby allowing Hb to bind oxygen and preventing its oxidation/denaturation to hemichromes [3]. Therefore, protection of the RBC membrane and Hb against oxidative damage largely depends on G6PD activity.

In G6PD deficiency ($G6PD^-$), failure to maintain normal levels of GSH due to decreased NADPH regeneration in the PPP results in insufficient anti-oxidant defence [1] and contributes to the etiology of acute hemolytic anemia following exposure to pro-oxidant drugs, infections and fava beans. Reactive oxygen species (ROS) are toxic in that they induce protein and lipid peroxidation, leading to intravascular RBC lysis and/or extravascular RBC clearance by macrophages [3]. G6PD deficiency represents the most common human enzyme genetic defect with a global prevalence of 5% and more than 400 million of people affected worldwide [4]. The prevalence of G6PD deficiency varies among ethnic groups being most common in Sub-Saharan Africa, Middle East and Asia (4.7–7.5%) compared to Americas and Europe (mostly Mediterranean area, 3.5–3.9%). Consistent with a probable protective effect on malaria infection, the distribution of $G6PD^-$ individuals correlates with the global distribution of malaria. More than 200 mutations and 400 biochemical variants have been identified [5]. The World Health Organization classifies the genetic variants of G6PD into five classes based on enzyme activity and clinically presented anemia. Classes I and II comprise variants that are related with less than 10% of the normal G6PD activity and chronic, nonspherocytic hemolytic anemia or intermittent hemolysis, respectively. Class III is a state of mild deficiency (10–60% of the normal activity, hemolysis with stressors only) while class IV is a non-deficient variant. Finally, class V variants exhibit increased enzyme activity and no clinical signs [6].

Oxidative injuries may play an important role in the evolution of the RBC storage lesion [7]. In the light of the increased susceptibility of $G6PD^-$ erythrocytes to oxidative stress-induced destruction, several investigators have proposed that the oxidative storage lesion can be amplified by the G6PD enzymopathy [8] and that these putative storage lesions condition the RBC for enhanced destruction after transfusion [3,9,10]. However, very few investigations have been conducted on the preservation of G6PD deficient cells and their post-transfusion viability. According to the World Health Organization's guidelines [11], blood can be accepted from $G6PD^-$ individuals without a history of hemolysis; however, their blood is not suitable for intrauterine transfusion, neonatal exchange transfusion or for patients with G6PD deficiency.

In the light of these observations, we assessed the storage quality of $G6PD^-$ RBCs through physiological, electron microscopy and omics approaches. In addition, we examined the *in vitro* reactivity of $G6PD^-$ donor or recipient blood under conditions mimicking the post-transfusion environment. While clinical evidence is awaited, we collected for the first time laboratory evidence suggesting that packed red cells from $G6PD^-$ individuals might store well in relation to energy and morphology parameters. However, $G6PD^-$ RBCs are more susceptible to oxidative lesions and lysis in a recipient-simulating context when compared with G6PD sufficient RBCs, questioning their suitability as routine donors.

2. Materials and methods

2.1. Chemicals and reagents

Following reagents (suppliers) were used in this study: 5-(and-6) chloromethyl-2′,7′-dichloro-dihydro-fluorescein diacetate, acetyl ester (CMH_2DCFDA) and fluorescent calcium indicator Fluo-4 AM (Invitrogen, Molecular Probes, Eugene, OR); Electron microscopy grade glutaraldehyde and osmium tetroxide (Serva, Heidelberg, Germany); Phycoerythrin (PE)-Annexin V (AnnV) Detection Kit I and fluorescein isothiocyanate (FITC)-conjugated anti-CD235a (BD Pharmingen, USA); Oxyblot detection kit (Millipore, Temecula, CA); HRP-conjugated antibodies to rabbit IgGs and ECL Western blot detection kit (GE Healthcare, Buckinghamshire, UK); Western lighting Plus ECL (Perkin Elmer, CA, USA); Bradford protein assay (Bio-Rad, Hercules, CA); Common chemicals (Sigma-Aldrich, Munich, Germany); LTRC blood bags (Haemonetics, MA, USA).

2.2. Blood collection and processing

Nine male, 22–30 years old $G6PD^-$ (class II Mediterranean variant, a common G6PD variant in Greece [8,12]) and 9 gender- and age-matched normal ($G6PD^+$) regular blood donors were recruited. All of them were Caucasians (of Greek origin). For the *in vivo* evaluation, venous blood was collected into EDTA or citrate vacutainers. RBC storage capacity was evaluated in citrate-phosphate-dextrose (CPD)/saline-adenine-glucose-mannitol (SAGM) log4 leukofiltered units (Haemonetics, MA, USA) throughout the storage period at 4 °C [13]. RBCs concentrates were prepared by centrifugation in an automated blood processing device (Compomat G4, Fresenius HemoCare) before 100 ml of SAGM solution (0.9 g dextrose monohydrate, 0.877 g sodium chloride, 0.0169 g adenine, 0.525 g D^- mannitol) was added. The volumes, total Hb content and hematocrit (Hct) of the RBC units were similar for the $G6PD^+$ group and the $G6PD^-$ group (363 ± 15 *versus* 358 ± 12 ml, 67.4 ± 6.5 *versus* 74.0 ± 4.2 g and 56.5 ± 2.5 *versus* 60.4 ± 5.2%, respectively). Samples were collected aseptically after 2 days of storage and weekly thereafter. The study was approved by the Research Bioethics and BioSecure Committee of the Faculty of Biology/NKUA. Investigations were carried out upon signing of written consent, in accordance with the principles of the Declaration of Helsinki.

2.3. Hematological and physiological analyses

Hematological analysis was performed using the Sysmex K-4500 automatic blood cell counter (Roche) for the estimation of RBC indexes (Hb concentration; hematocrit, Hct; mean corpuscular volume, MCV; mean corpuscular Hb, MCH; mean corpuscular Hb concentration, MCHC; and RBC distribution width, RDW). Biochemical analysis of triglycerides, cholesterol, low density lipoproteins (LDL), high density lipoproteins (HDL), iron (Fe), atherogenic index, total billirubin, aspartate transaminase (AST), alanine aminotransferase (ALT), potassium (K^+), sodium (Na^+) and ferritin was performed by using the automatic analyzers Hitachi 902, 9180 and Elecsys Systems Analyzer (Roche).

Plasma free Hb was calculated following a method first described by Harboe [14]. Hb absorbance in cell-free supernatants was measured *versus* blank at 380, 415 and 450 nm by the formula: $2 \times OD415 - OD380 - OD450$ [13]. Mechanical fragility of

RBCs was measured spectrophotometrically in blood mixed with stainless steel beads and rocked in a rocker platform for 1 h. Free Hb was measured in the plasma against an un-rocked control as previously described [13,15].

ROS (before and after stimulation by 100 μM *tert*-Butyl hydroperoxide (tBHP) or 2 mM diamide (20 min/25 °C and 45 min/37 °C, respectively) and calcium accumulation in RBCs at 1% Hct were calculated by fluorometry (VersaFluor Fluorometer System, Bio-Rad, Hercules, CA) after using the membrane-permeable, redox-sensitive probe CM-H_2-DCFDA (10 μM final concentration) or the fluorescent calcium indicator Fluo-4 AM (2 μM final concentration), respectively, according to the manufacturer's guidelines with minor modifications as previously described [16].

RBC morphology was evaluated by scanning electron microscopy (Philips SEM515) [16]. Classification of cell transformations was achieved by using the standard criteria with minor modifications [17,18]. Phosphatidylserine (PS) exposure on RBCs was estimated by multicolor flow cytometry using 2.5 μl of PE-AnnV and 2.5 μl of FITC-conjugated anti-CD235. The samples ran within 30 min in a FACScan flow cytometer (Beckton Dickinson) using CELL Quest Software (Becton Dickinson, San Jose, CA) and isotype matched FITC-antibodies.

RBCs were isolated by the method of Beutler [19], hemolysed in 5 mM sodium phosphate buffer (pH 8.0) containing protease inhibitor cocktail and membrane fractions were prepared [16]. For the measurement of carbonylated proteins, purified RBCs plasma membrane proteins were processed using the Oxyblot detection kit as per manufacturer's specifications (Millipore, Temecula, CA) with minor modifications as previously described [20]. The relative membrane expression of each component was estimated by scanning densitometry.

2.4. Omics analyses

Metabolomics analyses were performed as previously reported [21,22]. Briefly, 100 μl stored RBCs were collected on a weekly basis, extracted at 1:6 dilutions in methanol:acetonitrile:water (5:3:2), vortexed and centrifuged at 10,000*g* for 15 min at 4 °C to pellet proteins, prior to analysis of the supernatants by UHPLC-MS (ultimate high pressure liquid chromatography-mass spectrometry, Ultimate 3000RSLC-QExactive, Thermo Fisher) [21,22]. For metabolomics analyses 20 μL of samples extracts were injected into an UHPLC system and run on a a Kinetex XB-C18 column (150 × 2.1 mm i.d., 1.7 μm particle size – Phenomenex, Torrance, CA, USA) using either a 3 min isocratic flow at 250 μl/min (mobile phase: 5% acetonitrile, 95% 18 mΩ H_2O, 0.1% formic acid) or a 9 min gradient from 5 to 95% organic solvent B (mobile phases: A = 18 mΩ H_2O, 0.1% formic acid; B = methanol, 0.1% formic acid). The UHPLC system was coupled online with a QExactive system (Thermo, San Jose, CA, USA), scanning in Full MS mode (3 min method) or performing acquisition independent fragmentation (AIF - MS/MS analysis – 9 min method) at 70,000 resolution in the 60–900 *m/z* range, 4 kV spray voltage, 15 sheath gas and 5 auxiliary gas, operated in negative and then positive ion mode (separate runs).

For proteomics, RBC units (n = 6 G6PD$^-$ and n = 3 control) were sampled at storage days 2, 21 and 42 and RBC membranes were isolated [16]. Microvesicles (microparticles, MPs) were sorted from the supernatants of G6PD$^-$ (n = 3) and control (G6PD$^+$) units (n = 2) on day 42 of storage, through a three step high-speed centrifugation protocol at 37,000*g* for 1 h at 4 °C [23], and proteomics analyses were performed through a GeLC-MS approach [24]. In brief, 30 μg of pooled samples per each time point (days 2, 21, 42 RBC membranes and day 42 vesicles) were loaded into a 4–12% gradient 1D-SDS-PAGE gel and visualized with Coomassie staining. Twelve bands were cut for each lane, trypsin digested upon reduction with dithiothreitol and alkylation with iodoacetamide of unmodified cysteine residues, and run through nano-UHPLC-MS/MS (nano Easy LC II and Orbitrap Velos, Thermo Fisher) operated in positive ion mode. Raw data files were converted into. mgf files which were then merged using an in-house script. Error tolerant searches were done using Mascot (v. 2.4) against the human UniprotKB database (release date 2015.1.8), including decoy sequences. Relative quantitation was computed by spectral counting of peptide identifications meeting our confidence threshold (expectation value < 0.05).

2.5. In vitro model of transfusion

An *in vitro* transfusion-associated stress model was designed to simulate *in vivo* body temperature and effects of transfusion of stored G6PD$^-$ RBC units in healthy recipients. To that purpose, we incubated G6PD$^-$ or G6PD$^+$ RBCs or supernatant stored for 21, 30 or 42 days in fresh plasma or RBCs from ABO matched healthy volunteers (n = 3), respectively. Then, in order to examine the suitability of G6PD deficient subjects as recipients of stored RBC units from healthy donors, we incubated fresh (non-stored) G6PD$^-$ (grade II, Mediterranean variant, n = 3) or G6PD$^+$ (n = 3) RBCs or plasma with stored (21, 30 or 42 days old) supernatants or RBCs from ABO matched healthy donors. For both reconstitutions, RBC samples were diluted at 10–40% Hct in supernatant or fresh plasma generated by centrifugation of aseptically sampled aliquots of packed RBC units or fresh blood, respectively, at 2500*g* for 15 min at 4 °C. The reconstituted RBCs were incubated overnight at 37 °C in 5% CO_2-air along with aliquots of fresh (total) blood or packed RBC units without reconstitution. Measurements for hemolysis, intracellular ROS, mechanical fragility and PS exposure (see above) were performed immediately after the 24 h-incubation in mixed *versus* unmixed samples. Although measurements at 30% Hct are shown, preliminary experiments performed at various volume ratios of RBCs and supernatant/plasma showed that the profile of modifications is similar, despite variation in the numerical data.

2.6. Statistical analysis

All experiments have been performed in triplicate, unless otherwise stated. For statistical analysis, the Statistical Package for Social Sciences (IBM SPSS; version 22.0 for Windows) was used. Inter-group differences were evaluated by one-way ANOVA. Significance was accepted at $P < 0.05$ by using adjustment to unequal groups of data. Given the well-established significant inherent inter-donor biological variation in storage quality of RBCs [25,26], a statistical analysis for outliers was performed to minimize false discovery rate that is associated with the small size of the cohort involved. For omics analyses, relative quantitation, partial least square-discriminant analyses, hierarchical clustering analyses and other elaborations were performed with the software Excel (Microsoft, Redmond, CA, USA) and GraphPad (Prism, La Jolla, CA, USA).

3. Results

3.1. G6PD deficient donors exhibit normal hematologic profile but slightly variable RBC physiological parameters in vivo

G6PD$^-$ volunteers displayed normal hematologic and serum biochemical profiles (Table 1) and normal intracellular ROS accumulation *in vivo* (Non-stored, NS bar in Fig. 1A). However, borderline aberrations in serum lipids, ferrum and atherosclerosis indexes were detected in 30% of G6PD$^-$ subjects (Table 1).

Table 1
Hematologic and biochemical profile of G6PD deficient (G6PD$^-$, n=6) and control (C, n=3) donors *in vivo* and during storage of RBC units in CPD-SAGM.

	In vivo		Day 14		Day 28		Day 42	
	C	G6PD$^-$	C	G6PD$^-$	C	G6PD$^-$	C	G6PD$^-$
G6PD activity (IU/g Hb)	11.37 ± 2.87	1.44 ± 1.02*	N/D	N/D	N/D	N/D	8.20 ± 0.50	0.73 ± 0.71*
RBCs (x10^6/μl)	5.23 ± 0.57	5.07 ± 0.22	6.25 ± 0.58	6.39 ± 0.63	6.05 ± 0.59	6.16 ± 0.62	5.80 ± 0.37	5.74 ± 0.58
Hb (g/dL)	14.3 ± 1.8	15.2 ± 0.7	18.2 ± 0.9	20.4 ± 1.8	17.9 ± 1.0	20.0 ± 1.8	17.6 ± 1.1	19.6 ± 1.8
MCV (fL)	81.50 ± 4.3	89.8 ± 3.7*	86.9 ± 4.5	93.8 ± 4.2	87.60 ± 5.4	94.48 ± 3.9	89.10 ± 6.5	97.93 ± 4.3
MCH (pg)	27.40 ± 3.90	29.88 ± 1.52	29.12 ± 2.01	31.92 ± 1.55	29.59 ± 1.94	32.61 ± 1.52	30.34 ± 2.45	34.28 ± 1.66
MCHC (g/dL)	33.70 ± 1.61	33.12 ± 0.68	33.52 ± 1.42	34.01 ± 0.83	33.77 ± 1.41	34.52 ± 0.75	34.04 ± 0.93	35.01 ± 1.15
RDW (%)	12.4 ± 0.9	11.8 ± 0.4	14.7 ± 1.2	14.1 ± 0.4	15.5 ± 1.4	14.7 ± 0.4	16.8 ± 1.1	15.5 ± 0.5
RET (%)	2.05 ± 0.60	1.04 ± 0.29	–	–	–	–	–	–
HbF (%)	0.60 ± 0.33	0.30 ± 0.54	N/D	N/D	N/D	N/D	0.50 ± 0.22	0.58 ± 0.46
HbA$_{1c}$ (%)	5.00 ± 0.19	4.40 ± 0.25	N/D	N/D	N/D	N/D	7.10 ± 0.35	6.42 ± 0.26
HbA$_2$ (%)	2.70 ± 0.65	2.50 ± 0.38	N/D	N/D	N/D	N/D	2.70 ± 0.79	2.48 ± 0.46
Cholesterol (mg/dL)	160 ± 55.0	187.0 ± 36.3	N/D	N/D	N/D	N/D	N/D	N/D
HDL (mg/dL)	48.0 ± 6.3	46.3 ± 16.0	N/D	N/D	N/D	N/D	N/D	N/D
LDL (mg/dL)	91.0 ± 55.0	119.2 ± 30.0	N/D	N/D	N/D	N/D	N/D	N/D
Triglycerides (mg/dL)	107.0 ± 27.0	108.2 ± 53.7	N/D	N/D	N/D	N/D	N/D	N/D
Atherogenic index	3.3 ± 0.8	4.5 ± 1.0	N/D	N/D	N/D	N/D	N/D	N/D
Iron (mg/dL)	106.0 ± 42.0	152.7 ± 62.2	N/D	N/D	N/D	N/D	N/D	N/D
Ferritin (ng/dL)	148.0 ± 50.3	73.5 ± 57.0	N/D	N/D	N/D	N/D	N/D	N/D
Uric acid (mg/dL)	5.60 ± 1.25	5.00 ± 1.03	N/D	N/D	N/D	N/D	N/D	N/D
Total bilirubin (mg/dL)	0.50 ± 0.37	0.90 ± 0.52	N/D	N/D	N/D	N/D	N/D	N/D
AST (U/L)	23.0 ± 4.6	17.2 ± 3.6	N/D	N/D	N/D	N/D	N/D	N/D
ALT (U/L)	37.0 ± 6.9	26.0 ± 5.2	N/D	N/D	N/D	N/D	N/D	N/D

N/D=not determined.

* $P < 0.05$ *versus* control.

Moreover, over-susceptibility to ROS generation following treatment with tBHP or diamide (Fig. 1B and C) and over-expression of carbonylated membrane proteins (Fig. 1D) were detected in G6PD$^-$ RBCs. MCV (Table 1), PS exposure (Fig. 1E) and RBC mechanical fragility (see Fig. 2 in Ref. [27]) were also significantly higher ($P < 0.05$), though within normal range, in the G6PD$^-$ RBCs *in vivo* compared to controls.

3.2. The stored G6PD$^-$ RBCs demonstrate acceptable levels of storage lesion-associated parameters and better morphology compared to controls

The stored G6PD$^-$ RBCs exhibited a trend for higher MCV and mean corpuscular Hb (MCH) compared to the G6PD$^+$ RBCs throughout the storage period (Table 1). G6PD enzymatic activity decreased during the storage in both groups of RBC units (50% *versus* 30% loss on day 42 for G6PD$^-$ and G6PD$^+$ RBCs, respectively). However, the final level was still within normal range (> 7.1 IU/g Hb) in control RBC units. G6PD activity *in vivo* correlated with G6PD activity *ex vivo* throughout the storage period ($P < 0.05$, R=0.841) as well as with the intracellular Hb levels of day 42 stored G6PD$^-$ RBCs ($P < 0.05$, R=0.906).

Comparable levels of endogenous ROS (Fig. 1A) but significantly higher tBHP- or diamide-induced ROS generation (Fig. 1B and C) were observed in the G6PD$^-$ compared to the G6PD$^+$ RBCs during the storage period. PS exposure and RBC membrane protein carbonylation were also higher in the stored G6PD$^-$ RBCs compared to controls on day 21 or 28 onwards, respectively (Fig. 1D and E), consistent with the basal levels.

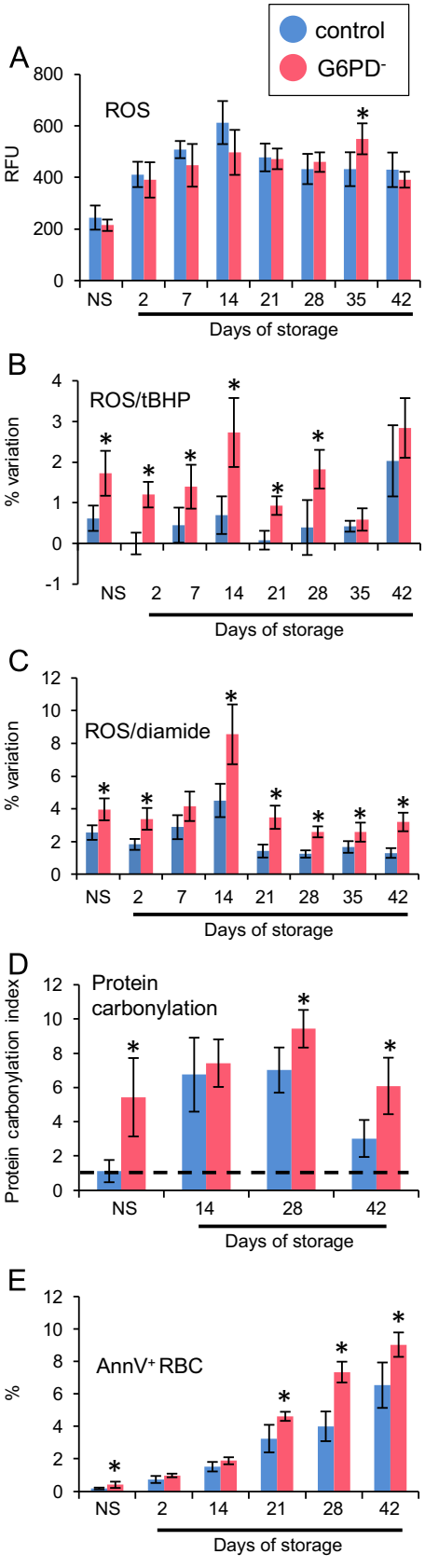
Morphologic examination revealed a progressive accumulation of reversible and irreversible RBC shape modifications over storage (Fig. 2A). Notably, the percentage of spheroechinocytes and other types of non-reversibly modified RBCs was significantly lower in the G6PD$^-$ units compared to control units after the middle of the storage period (Fig. 2B). In a similar way, intracellular calcium accumulation was constantly lower in the stored G6PD$^-$ cells, although the difference reached significance only on day 42 samples (Fig. 2C). As expected, the RBC osmotic and mechanical fragility increased during the storage, however, at similar rate in G6PD$^-$ and G6PD$^+$ cells (see Fig. 2 in Ref. [27]).

A trend for lower average free (oxy-) Hb levels was observed in the G6PD$^-$ units compared to the control (Fig. 3A) that was inversed following incubation of stored RBCs for 24 h at 37 °C (Fig. 3B). In contrast, supernatant potassium concentration was significantly higher in G6PD$^-$ units *versus* control at the end of the storage period (8.8-*versus* 7.1-fold increase, respectively, compared to the basal levels). Supernatant sodium (124.0 ± 3.5 *versus* 126.0 ± 3.2 mM, respectively), microparticles accumulation and total antioxidant capacity (see Figs. 3 and 4 in Ref. [27]) exhibited no significant difference between the control and G6PD$^-$ units. Supplementation of RBC units under examination with N-acetylcysteine (2.5 mM final concentration) equally improved redox parameters in cells and supernatants of both groups. On the other hand, fragility and potassium loss were ameliorated in the control stored cells but not in G6PD deficient stored cells (see Fig. 6 in Data in Brief - Ref. [27]).

3.3. G6PD$^-$ cells promote glucose fluxing through the Embden Meyerhof pathway at the expenses of anti-oxidant potential

Metabolomics analyses were performed through UHPLC-MS to monitor alterations of energy and redox metabolism during storage of control and G6PD$^-$ cells. Control results were consistent with previous findings on metabolic alterations of RBCs stored in SAGM [28–31] and other additive solutions [21,32,33], showing a progressive loss of the RBC capacity to fuel the oxidative phase of the PPP after the second week of storage, consistently with the measured storage-dependent reduction in G6PD activity (Fig. 4). In G6PD$^-$ cells, PPP was depressed since the beginning of the storage period, as inferred from steady state levels of final products of this pathway (*i.e.* significantly higher glucose 6-phosphate levels and lower ribose phosphate levels were observed at each time point – Fig. 4). This corresponded to increased glucose oxidation through the Embden Meyerhof pathway, promoting accumulation of glycolytic intermediates and products, including phosphoglycerate and pyruvate at each time point. On the other hand, lactate levels were

comparable between groups, as well as NADH, NAD^+ and NADH/NAD^+ ratios, suggesting alternative oxidation pathways than lactate dehydrogenase (LDH) in $G6PD^-$ cells (Fig. 4). The observed increase in basal levels of methemoglobin reductase (NADH-dependent cytochrome b5 reductase – Fig. 4) might explain this observation, as an alternative route to cope with increased oxidation of heme iron in $G6PD^-$ cells [34]. Indeed, decreased fluxes through NADPH-generating PPP also corresponded to decreased levels of reduced glutathione and reduced/oxidized glutathione ratios (GSH/GSSG), suggesting a potential impairment in total antioxidant capacity in $G6PD^-$ cells [34]. On the other hand, increased levels of glycolytic intermediates also corresponded to higher levels of adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (DPG), at least during early storage weeks (< 14 days).



3.4. Proteomics analysis indicated increases in oxidation/stress markers accumulation in RBC membrane and vesicles of $G6PD^-$ RBCs

Though stored $G6PD^-$ RBCs were characterized by better morphology, $G6PD^-$ RBC vesicles (microparticles) had proportionally higher levels of anti-oxidant enzymes (peroxiredoxins 1, 2 and 6 – Prdx; superoxide dismutase, *etc.*), hemoglobins (especially alpha and beta subunits) and stress-related markers (heat shock proteins, proteasome components b1, b2 and b5) (Fig. 5). Basal and storage-dependent membrane levels of Prdx2 were higher in $G6PD^-$ cells [34] and vesicles, expanding on previous findings on newborns [35]. Proteins in vesicles from both control and $G6PD^-$ cells [34] were mostly oxidatively modified, such as in the case of cysteine β94 of hemoglobin (Fig. 5). Of note, $G6PD^-$ vesicles were characterized by higher levels of components of the coagulation and complement cascades (Fig. 5).

3.5. In vitro model of transfusion suggests that G6PD deficient RBCs could be more susceptible to hemolysis and oxidative stresses within the recipient

We sought to examine the possible effects associated with the transfusion of G6PD deficient units by using an *in vitro* model of transfusion. To that purpose, we incubated $G6PD^-$ RBCs and supernatants stored for either 21, 30 or 42 days with fresh plasma and RBCs from healthy volunteers, respectively. Measurements were performed 24 h post mixing at 37 °C, as to simulate post-transfusion events in apparently healthy recipients. Hemolysis and ROS accumulation were observed when stored $G6PD^-$ RBCs were incubated with fresh healthy plasma, but not (or comparatively lower) for controls (Fig. 6A). This phenomenon was exacerbated in the presence of exogenous tBHP (days 21 and 30) and diamide (day 42) stimulation, as to simulate stress conditions often observed in critically-ill recipients. Under the same conditions, significant differences were also detected in the mechanical fragility of stored RBCs from $G6PD^-$ and $G6PD^+$ volunteers that exhibited a storage time-dependent deterioration in fresh, recipient plasma.

On the other side, when fresh RBCs from healthy volunteers were incubated with the supernatant of $G6PD^{-/+}$ units, a similar antioxidant effect of the supernatant was observed on "recipient" fresh, healthy RBCs (Fig. 6B). Long stored (> 21 days) $G6PD^-$ supernatants were less effective in preventing ROS generation

Fig. 1. Oxidative stress and cell clearance markers in G6PD deficient (n=6) and control ($G6PD^+$, n=3) RBCs *in vivo* (non-stored, NS) and during storage in CPD-SAGM. (A-C) Reactive oxygen species (ROS) accumulation before (A) and after stimulation by *tert*-BHP (ROS/tBHP, B) or diamide (ROS/diamide, C). Stimulated ROS were normalized to the pre-treatment values. RFU, relative fluorescence units. (D) RBC membrane protein carbonylation is significantly elevated in $G6PD^-$ donors *in vivo* and during storage. Values (immunoblotting results) were normalized (dashed lines) to the basal (NS) levels of the control cells. (E) Flow cytometry results of $AnnV^+$ RBCs. Percentage of PS-exposing RBCs is higher in $G6PD^-$ donors *in vivo* and after the middle of the storage period. $^*P < 0.05$ *versus* control; data shown as mean ± standard deviation.

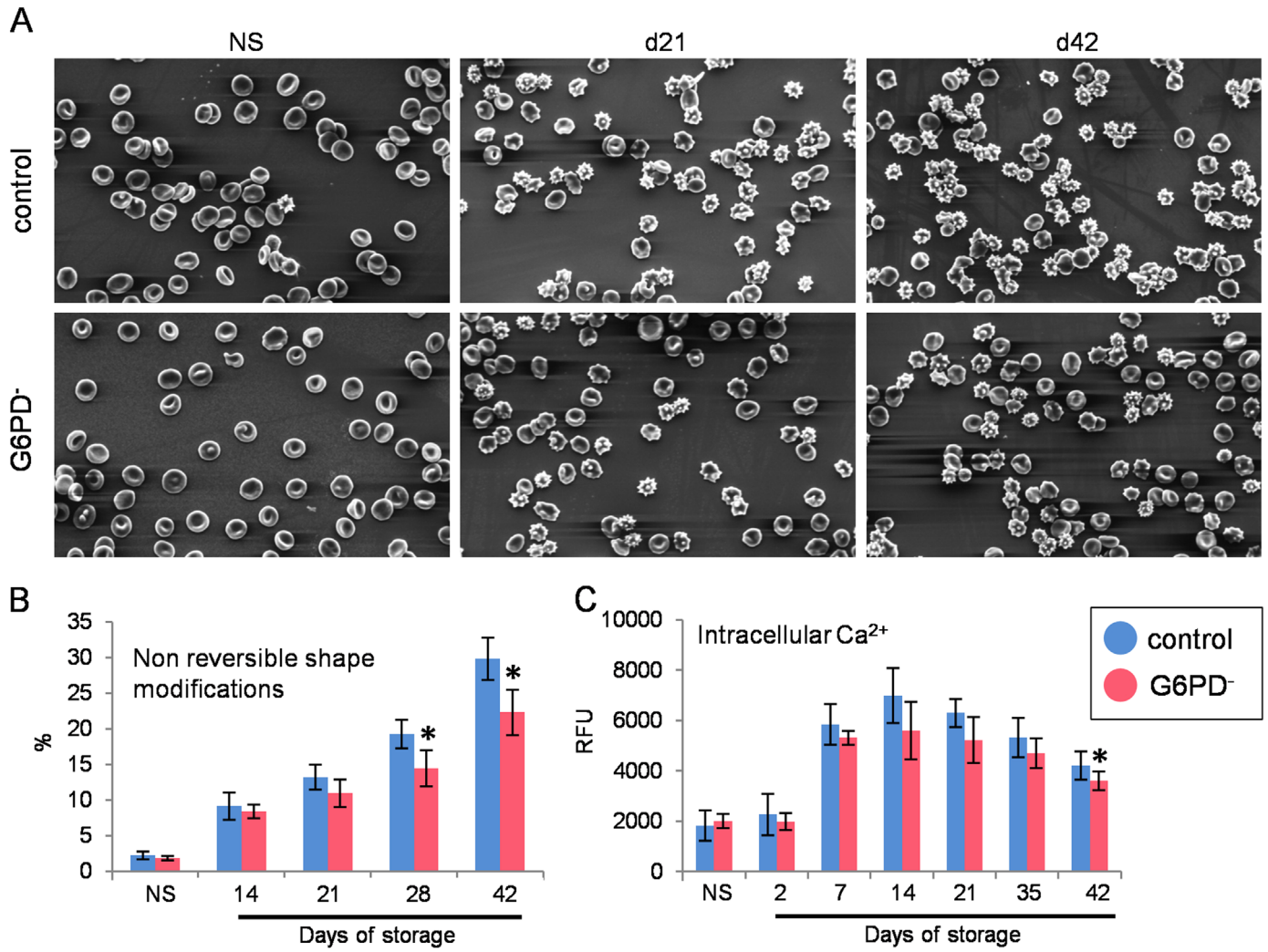


Fig. 2. G6PD deficient donors demonstrate lower levels of non-reversibly modified RBCs and intracellular calcium. (A) Representative scanning electron micrographs (Philips SEM515, magnification $\times 1000$) of RBCs from G6PD$^-$ and control donors before (NS, non-stored) and during storage days 21 (d21) and 42 (d42). (B) Percentage of non reversible shape modifications was significantly lower in G6PD$^-$ donors after the middle of the storage period. (C) G6PD$^-$ donors demonstrated a trend for lower levels of calcium accumulation during storage. RFU, relative fluorescence units. $^*P < 0.05$ *versus* control.

stimulated by tBHP in fresh healthy RBCs. Hemolysis of fresh RBCs was increased by both G6PD$^-$ and control supernatants collected from day 42 units. Notably, incubation of fresh "recipient" RBCs with either >21 day G6PD$^-$ or 42 day control supernatants increased PS exposure on recipient cells (Fig. 6B).

The same *in vitro* model of transfusion was used to assess whether G6PD deficient subjects could represent good recipients of stored RBC units. To this purpose, fresh G6PD$^-$ or control RBCs and plasma were mixed (for 24 h at 37 °C) with stored (for 21, 30 or 42 days) RBCs from healthy donors. Fresh ("recipient") plasma from G6PD$^-$ subjects protected the stored G6PD$^-$ sufficient RBCs from endogenous or stimulated ROS and PS externalization (on day 21) better than fresh G6PD$^+$ plasma (Fig. 7A). On the other hand, the "recipient" G6PD$^-$ RBCs were more efficient than healthy RBCs in coping with oxidative stress elicited from the exposure to the supernatant of RBC units stored for various periods, either in presence or absence of the stress enhancer tBHP (Fig. 7B). The same was true for the mechanical fragility of the recipient cells.

4. Discussion

Guidelines on assessing G6PD$^-$ donor suitability for blood donation demand answers to two key questions:

(1) Are G6PD deficient RBCs more susceptible to processing and storage lesion than erythrocytes from healthy donors?
(2) Can transfusion of G6PD$^-$ blood promote untoward consequences to patients, either as a result of the G6PD$^-$ pathophysiology or as secondary effect of the aforementioned storability issue?

While clinical trials that are currently underway and will contribute to clarify these issues are awaited [3], our studies provide preliminary basic science laboratory evidence to address in part both of these concerns. Comparative results on stored RBCs from G6PD$^-$ or healthy donors revealed that G6PD$^-$ cells might store well in relation to morphology, calcium and energy related parameters, though at the expenses of a compromised anti-oxidant system. We observed that storage *per se* seemed to represent a "tolerable"-level of stress for the G6PD$^-$ cells, which can effectively challenge the storage-associated oxidative stimuli in presence of a strong antioxidant supernatant environment. G6PD$^-$ RBCs are not only characterized by similar levels of in-bag hemolysis, fragility, membrane exovesiculation and ROS/calcium accumulation during the storage, but also by improved rheological properties (decreased spheroechinocytosis, trend for higher mean corpuscular Hb and volume) compared to the G6PD$^-$ sufficient donors.

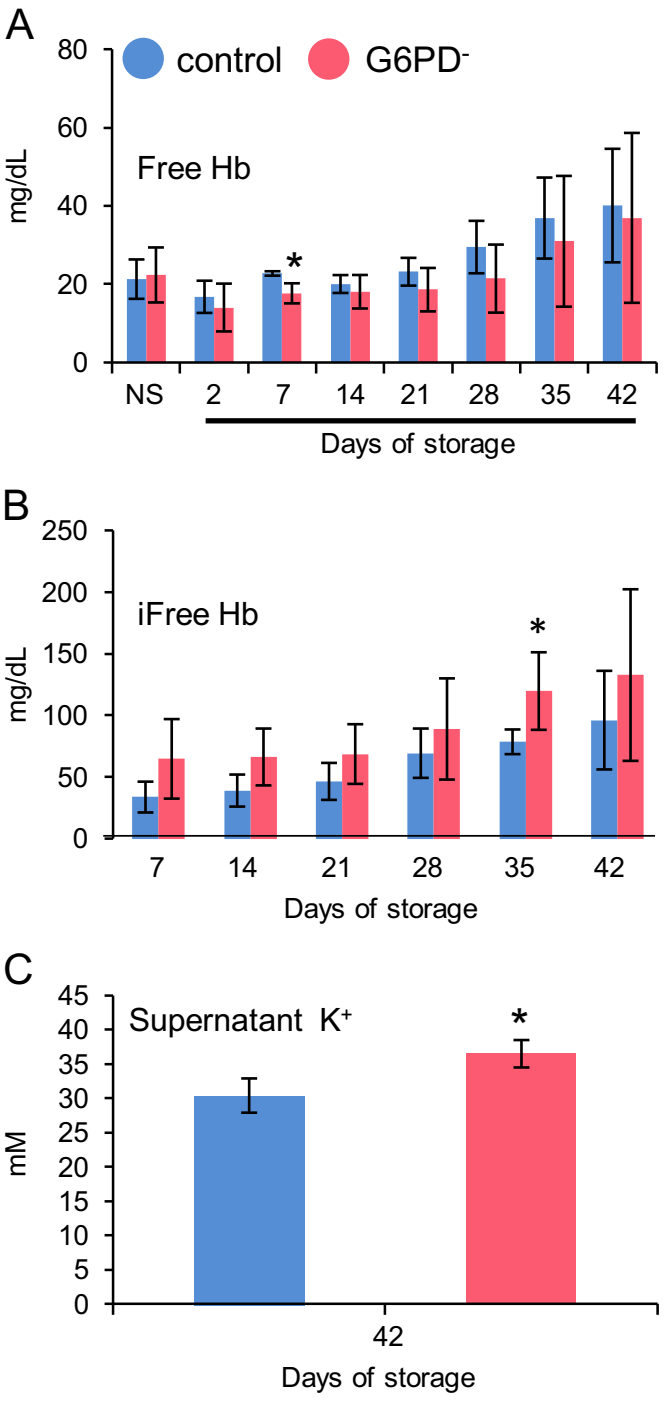


Fig. 3. Plasma and supernatant examination in G6PD$^{-/+}$ donors. Free Hb *in situ* (A) and following incubation for 24 h at 37 °C (iFree Hb, B) in the plasma (non-stored, NS) and the supernatant of the control (G6PD^{+}, n=3) and G6PD^{-} (n=6) RBC units. (C) Potassium concentration on the last day of storage. *$P < 0.05$ *versus* control.

Regarding spheroechinocytosis, it has been suggested that the irreversibly deformed RBCs comprise at least a fraction of the removal-prone erythrocytes [36]. Lower spheroechinocytosis in the G6PD^{-} units might be associated with the higher RBC volume and the lower intracellular calcium accumulation that are common characteristics of a younger erythrocyte population. Indeed, in agreement with previous reports [9,37], the G6PD^{-} RBCs were characterized by lower levels of the cell age marker HbA1c and significantly increased MCV (that characteristically decreases with RBC aging [38]) compared to the G6PD^{+} RBCs. In fact, not only spheroechinocytosis, but the somewhat altered storage lesion of G6PD deficient RBCs might be related to their shortened lifespan and hence younger mean age compared to control, as previously observed for the stored sickle RBCs [39]. RBCs age at the time of donation impacts upon the quality of stored RBC concentrates [40,41]. The long history of studying transfusion efficacy of stored "neocytes" (less dense and presumably youngest circulating RBCs) has shown that young RBCs exhibit higher post-transfusion survival rate than standard (of variable age) or old RBCs, an advantage that may translate into reduced blood requirements and transfusion-associated complications in chronically transfused patients [42]. Notably, (i) some beneficial characteristics of neocytes compared to "gerocytes" *in vivo* (*e.g.* high ATP content) are not preserved during storage [41,43], (ii) a variable response to neocyte transfusions has been reported among recipients [44] and (iii) more recent evidence has shown that neocyte transfusion is less advantageous than expected, and thus it is nowadays not practiced on a large scale for the long-term treatment of patients [45]. Despite this evidence, the above mentioned studies highlighted the functional relation of transfusion performance with *in vivo* age of RBCs that drives in part the storage lesion mechanism, including the *ex vivo* aging of RBCs.

Stored RBCs, however, degrade over time through more than one mechanism of cellular injury [16]. Apart from RBC aging, redox and metabolome homeostasis closely interact with each other in response to donor-related factors, storage time and strategies to determine the functional and structural integrity of the preserved RBCs [46]. Indeed, a further tentative explanatory mechanism for the observed lower calcium levels in G6PD^{-} RBCs at the end of the storage period, is attributable to the necessity of these cells to metabolize glucose through the Embden-Meyerhof pathway at the expenses of PPP, thereby privileging energy homeostasis (higher ATP and DPG levels were observed during early storage) at the expenses of anti-oxidant potential. Improved energy homeostasis might in turn contribute to reduced calcium ion permeability and, subsequently, improved morphology [47]. Disordered calcium homeostasis with impaired calcium ATPase activity (hypothetically associated with increased calcium permeability), increase in intracellular calcium levels and leakage of RBC potassium have been previously described during acute hemolytic crisis in favism [48]. However, occurrence of these events is infrequent in G6PD deficient non-hemolytic healthy individuals *in vivo* [48]. Therefore, alterations of other transport systems (*e.g.* K-Cl symport [49]) rather than Gardos might justify the loss of erythrocytic potassium, moderately higher in the G6PD^{-} cells at the end of the storage period. Of note, ATP-dependent calcium-independent hemolytic phenomena have been previously documented in fresh and stored RBCs [50].

Though energy metabolism is a key regulator of RBC morphology and survival *in vivo* [51], oxidative stress orchestrates a significant part of the RBC storage lesion. Stored RBCs accumulate ROS, oxidative biomarkers and oxidative lesions to lipid and protein components over storage time [16,52]. As storage progresses, a decrease in GSH stability is observed that correlates with 24-h post transfusion recovery [53]. Our results indicate that, despite impairment of the antioxidant systems (impaired glutathione and thiol homeostasis, increased membrane carbonylation), G6PD^{-} cells may still be equipped to cope with the oxidative stress arising during routine storage. To partially explain this phenomenon, we noted that the supernatants from both control and G6PD^{-} units exert a strong antioxidant effect on stored (and fresh) RBCs in comparison to fresh plasma. However, when the stored cells are incubated with fresh plasma, ROS accumulation sharply increased in G6PD^{-} stored cells compared to normal RBCs stored for the same period. Moreover, anti-oxidant pathways other than GSH may be up-regulated in G6PD^{-} RBCs in comparison to control

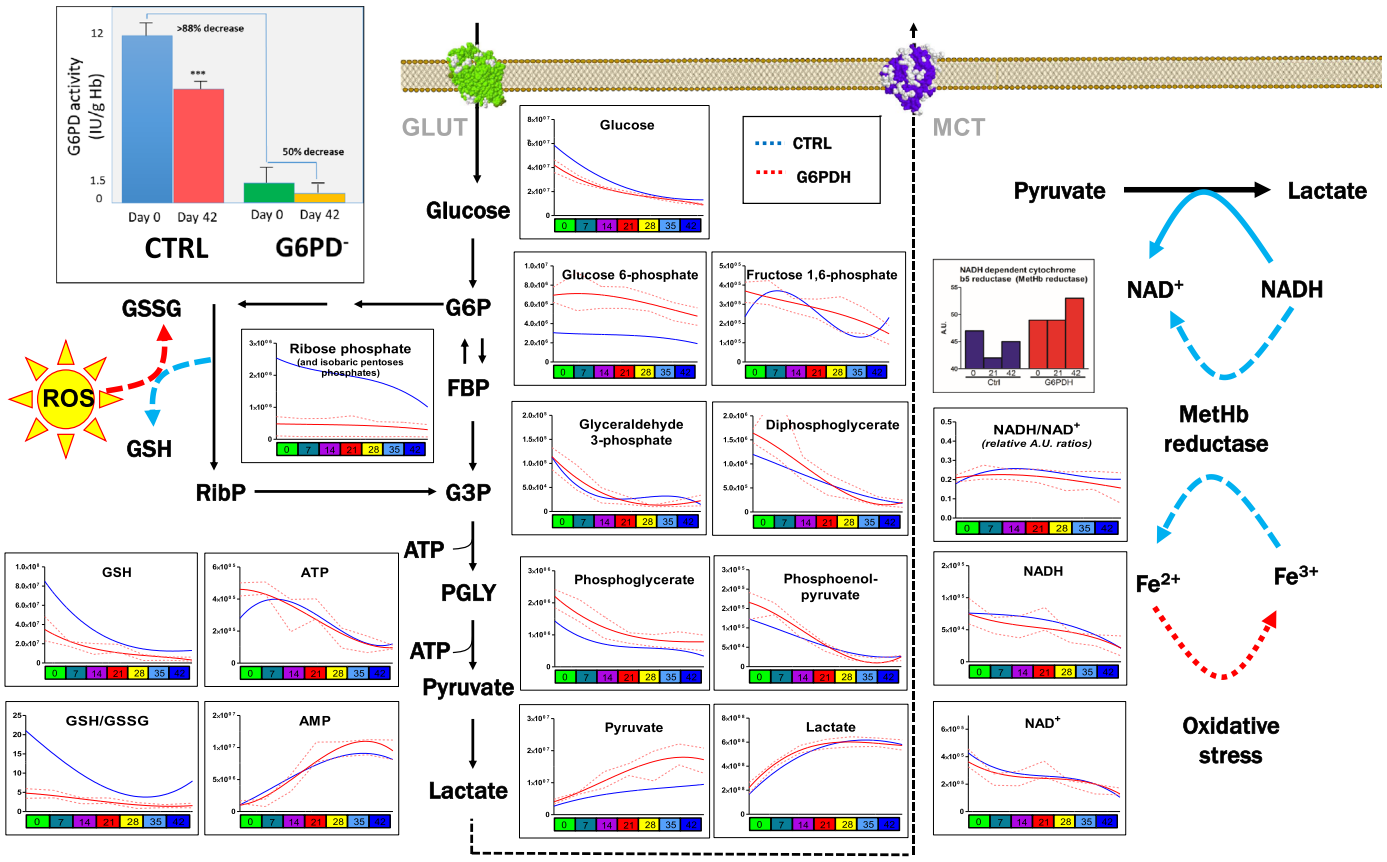


Fig. 4. Metabolomics analyses show increased fluxes through Embden-Meyerhof at the expenses of PPP and GSH homeostasis. G6PD activity is shown in control (CTRL, G6PD$^+$) and G6PD$^-$ RBCs before storage (Day 0) and at the end of the storage period (Day 42). This corresponded to a bottleneck at the glucose 6-phosphate level and constitutively lower levels of the PPP byproduct ribose phosphate. In turn, glutathione homeostasis was impaired (lower GSH and GSH/GSSG ratios), while ATP and DPG levels were better preserved at least during the first two-three storage weeks. NADH, NAD$^+$ and ratios were comparable in both groups, despite pyruvate levels and pyruvate to lactate ratios being significantly higher in G6PD$^-$ cells at each time point. This observation is consistent with alternative recycling routes for NADH from other enzymes than lactate dehydrogenase, such as the Fe^{3+} to Fe^{2+} cytochrome b5, methemoglobin reductase (constitutively higher in G6PD$^-$ cells as gleaned through proteomics analyses). Blue line: control; solid and dashed red lines: median ± interquartile range for G6PD$^-$ cells.

erythrocytes. Indeed, peroxiredoxin levels were relatively higher in G6PD$^-$ microparticles than in controls, suggesting an over-activated defense mechanism against oxidative stress in G6PD deficient RBCs - though clearer indications on the relevance of this observation will be obtained through the application of absolute quantitation proteomics approaches [54]. From a metabolic standpoint, levels of malate decreased faster in G6PD$^-$ RBCs than in controls (see Fig. 5 in Ref. [27]). Recently, it has been argued that RBCs can regenerate NADH from malate through malate dehydrogenase [31,33]. NADH regeneration through this mechanism may play a yet unexplored role in regenerating the metHb reductase battery in G6PD$^-$ RBCs.

On the basis of these findings, the G6PD$^-$ donors might be regarded as “good storers”, exhibiting acceptable RBC storability under routine storage conditions. However, after additional oxidant insult (tBHP or diamide), metabolic stress (see hemolysis at 37 °C), or reconstitution in G6PD$^+$ blood, the stored G6PD$^-$ cells “collapsed”, exhibiting higher levels of hemolysis, mechanical fragility and ROS accumulation compared to the G6PD sufficient cells. These conditions were chosen as to simulate viability of stored G6PD$^-$ cells when exposed to the post-transfusion environment. Indeed, hemolysis undermines 24 h post-transfusion RBC recovery and leads to recipient's burden with free Hb and Hb-degradation products, while externalized phosphatidylserine is a strong removal signal mediating erythrophagocytosis, adhesion to endothelium and thrombogenic events [55,56]. Moreover, cellular fragility represents a measure of the integrity of RBC membrane and hence of the ability of an intact cell carrying sub-lethal membrane damage (as a result of storage lesion or underlying pathophysiology) to withstand shear stresses. Previous studies have shown that sub-lethal damage of RBC can differ among RBC units exhibiting similar degree of in-bag hemolysis [15]. Consequently, a high fragility index reveals RBCs susceptible to lysis in a hostile environment where additional stresses are present. Finally, intracellular ROS accumulation orchestrates several molecular mechanisms in RBCs that end up in generation of senescent neoantigen, PS exposure at RBC surface, release of microparticles, membrane loss, cellular rigidity *etc.* [46].

In vitro models of transfusion that simulate post-transfusion context and transfusion-associated stresses under certain, controlled conditions have proved to be acceptable and valuable tools in giving information regarding the biochemical and physiological modifications that happen in stored RBC following their contact with recipient blood at body temperature [57]. Although they are characterized by lack of the RBCs natural environment, inability to probe extravascular hemolysis (addition of erythrophagocytosis assays, however, might compensate in part for this weakness) and probable *in vitro* artifacts compared to the *in vivo* state afforded by animal models [58], *in vitro* experiments offer the possibility to study large numbers of human samples and control for a number of variables confounding observations in the clinical settings within the framework of large scale randomized evidence based studies, such as RBC age, volume, and donor/recipient variability conditions. Moreover, preliminary results from *in vitro* studies, such as performed in the present model, may provide a molecular rationale to inform the interpretation of future human studies

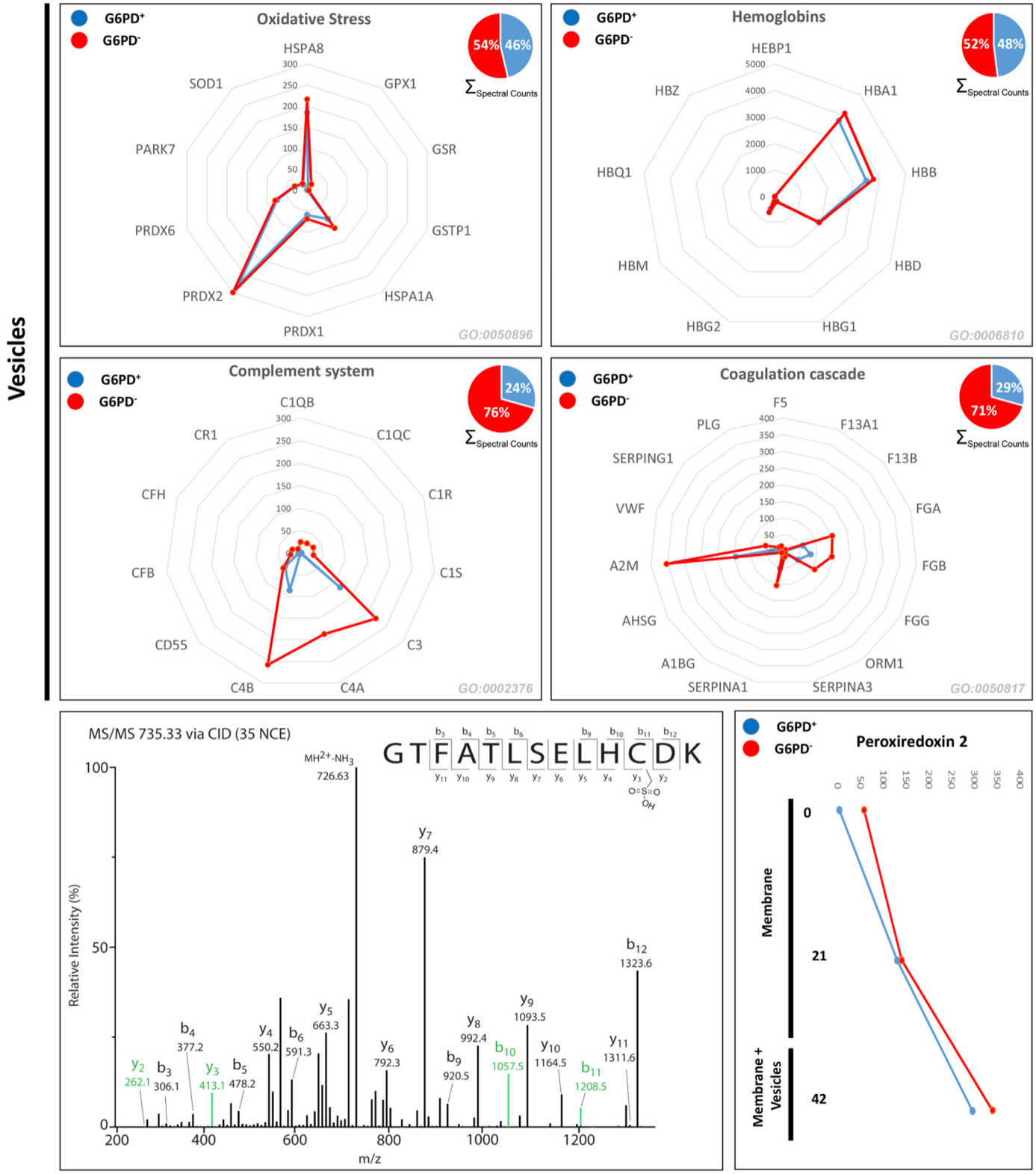


Fig. 5. Proteomics analyses of RBC membrane and vesicles (microparticles) from G6PD$^-$ and healthy donors indicate higher oxidative stress and increased pro-inflammatory and pro-coagulant potential of the former. Vesicle proteins were enriched for gene ontologies (GO) associated with biological functions and plotted as star diagrams showing relative quantitation values based upon spectral counts. Vesicles from G6PD$^-$ supernatants (storage day 42) were enriched with anti-oxidant enzymes, hemoglobins, complement and coagulation cascade components. Membrane and end storage vesicle levels of peroxiredoxin 2 were higher in G6PD$^-$ units. A representative nanoLC-MS/MS spectrum of oxidized Cysteine Beta93 of hemoglobin is shown from G6PD$^-$ vesicles after 42 days of storage.

investigating the linkage between RBC storage lesion and post-transfusion performance in specific recipient groups, *e.g.* patients under oxidative stress or inflammation.

G6PD$^-$ RBCs are extremely sensitive to oxidative damage to membrane proteins resulting in destabilization of the cell membrane [59]. Increased lipid peroxidation [60] and carbonylation of cytoskeletal protein components (including spectrin, ankyrin and band 4.1R) have been reported in G6PD deficient RBC [61]

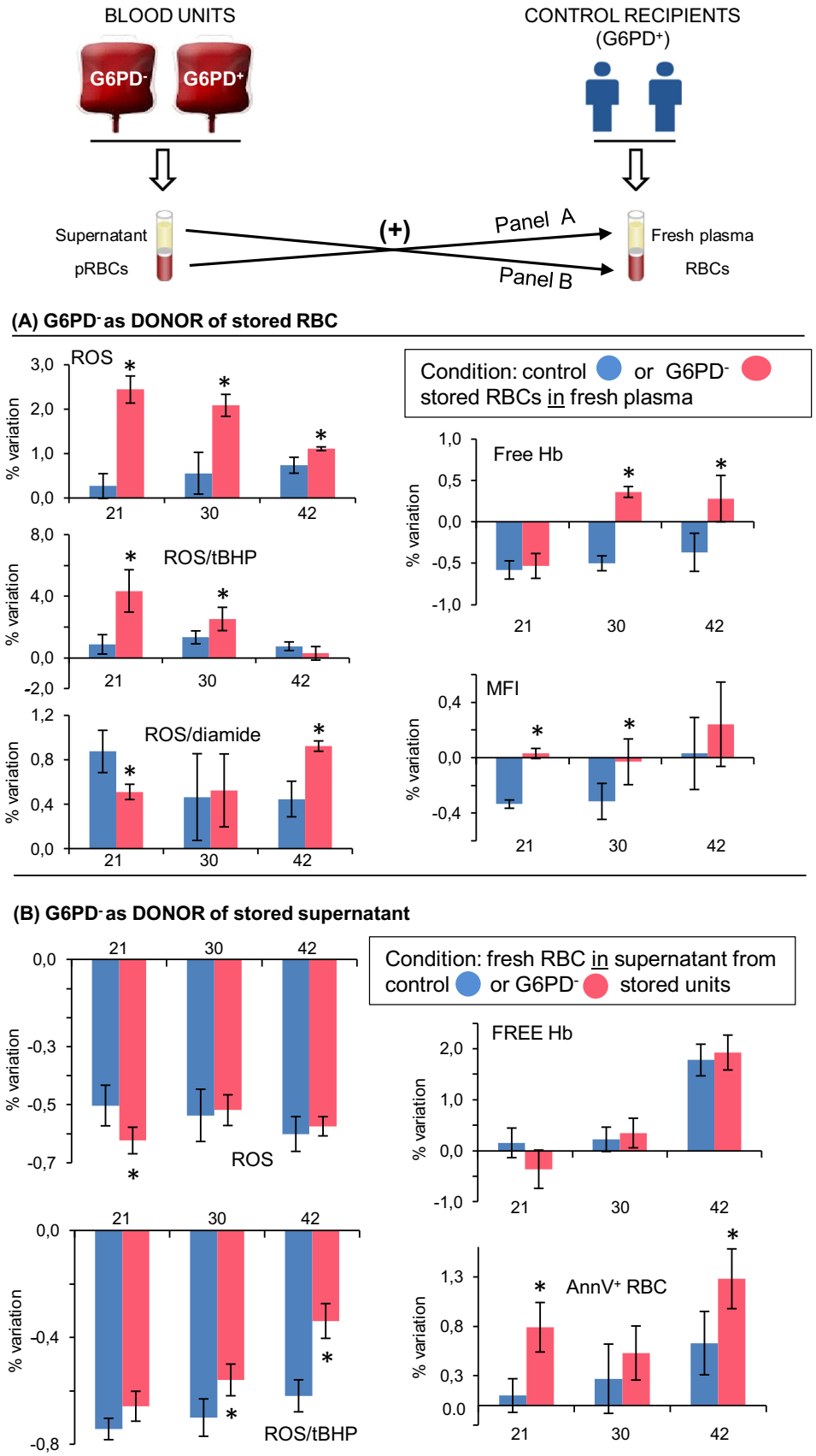


Fig. 6. Reconstitution experiments (*in vitro* model of transfusion) using stored blood from G6PD deficient donors. (A) Physiological measurements (intracellular ROS, hemolysis and RBC mechanical fragility) in G6PD$^-$ (n=6) and control (G6PD$^+$) (n=3) stored cells following incubation for 24 h at 37 °C with fresh plasma from ABO-matched healthy individuals (n=3), as described in the Materials and methods section. (B) Comparative graphs showing the effect of G6PD$^-$ or control stored supernatant on healthy, fresh (non-stored) RBCs regarding intracellular ROS, hemolysis and PS exposure levels. All values are normalized to those of total blood (fresh RBC and plasma) or RBC unit material (stored RBC and supernatant) treated at the same conditions (24 h at 37 °C). *$P < 0.05$ *versus* control samples.

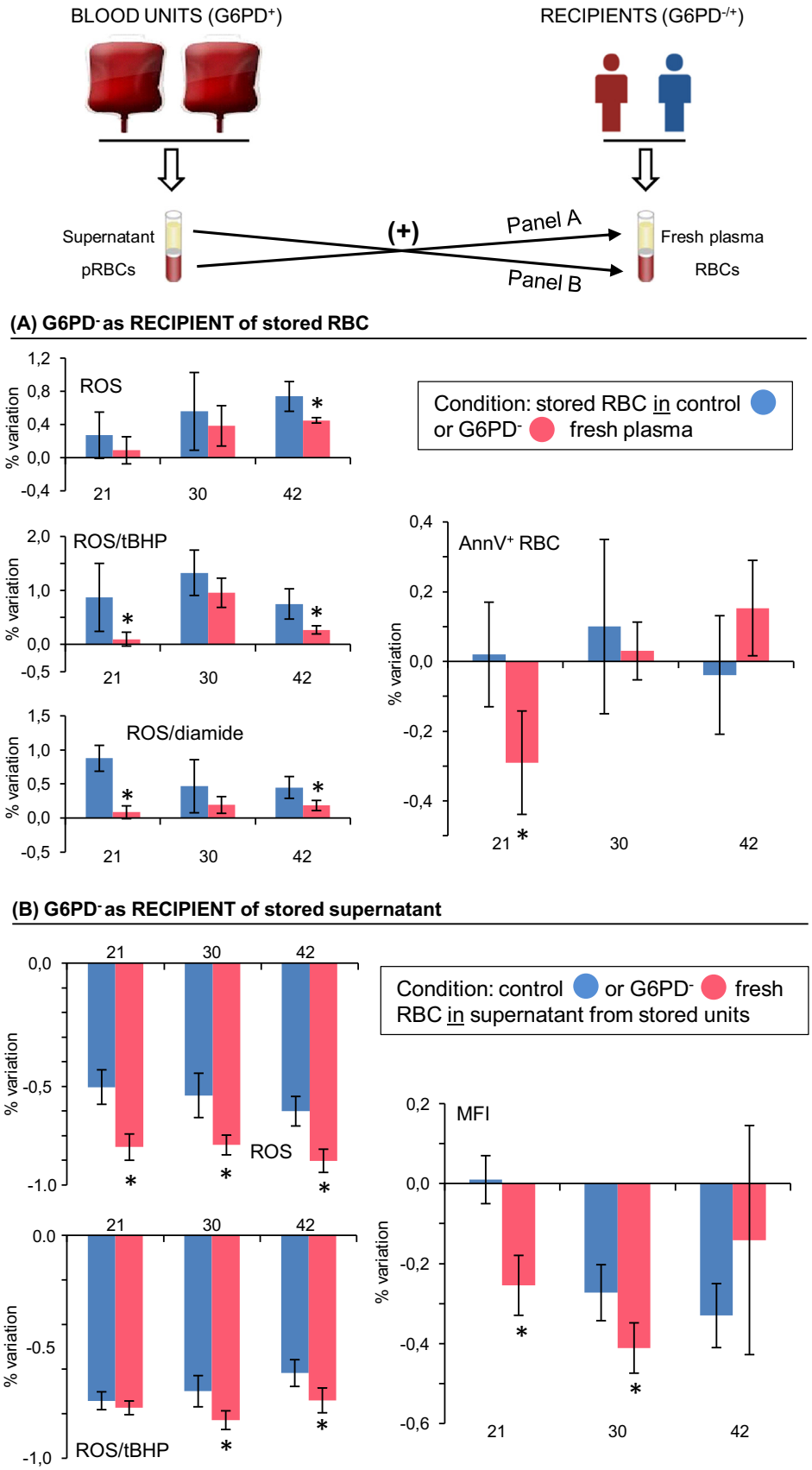


Fig. 7. Reconstitution experiments (*in vitro* model of transfusion) using G6PD$^-$ individuals as recipients of G6PD$^+$ stored blood. (A) Physiological measurements (intracellular ROS and PS exposure) in stored G6PD$^+$ RBC (n=3) following incubation for 24 h at 37 °C with fresh (non-stored) plasma isolated from ABO-matched grade II WHO G6PD$^-$ (n=3) and control (G6PD$^+$) (n=3) individuals, as described in the Materials and methods section. (B) Comparative graphs showing the effect of G6PD$^+$ stored supernatant on fresh (non-stored) G6PD$^-$ and G6PD$^+$ RBC regarding variation in intracellular ROS and mechanical fragility index (MFI). All values are normalized to those of total blood (fresh RBC and plasma) or RBC unit material (stored RBC and supernatant) treated at the same conditions (24 h at 37 °C). *$P < 0.05$ *versus* control donors.

compared to controls, resulting in severe RBC deformability issues in G6PD deficient patients [60]. In addition, oxidative stress is a strong inducer of eryptosis, a phenomenon previously observed in G6PD deficiency. G6PD$^-$ RBCs are more susceptible to PS exposure following osmotic, oxidative, calcium or energy depletion stress compared to control cells [62]. In agreement with previous reports [37], our G6PD$^-$ donors were characterized by higher (though non-pathologic) levels of PS exposure *in vivo* compared to controls, verifying both the previously shown slightly shortened RBC lifespan in G6PD deficiency [63] and the increased PS externalization in hemolytic patients [64]. Moreover, depletion of anti-oxidant defenses during storage was accompanied by increased PS externalization in stored G6PD$^-$ cells compared to G6PD$^+$ cells. The supernatant of the G6PD$^-$ units, that promoted PS externalization in fresh "recipient" RBCs, was enriched in extracellular potassium (K^+) and potentially pro-inflammatory and thrombogenic MPs. Indeed, our proteomics analyses detected higher levels of complement and coagulation cascade components in vesicles from G6PD$^-$ subjects in comparison to healthy controls, suggesting that units from this category of donors might promote untoward inflammatory consequences potentially associated with transfusion (*e.g.* transfusion-related acute lung injury, TRALI [65]) and pose potential threats [66] to certain categories of recipients (*e.g.* hypercoagulable trauma patients [67]).

The aforementioned observations were dependent on storage duration, consistent with previous observations in G6PD deficiency, reporting that old RBCs showing the lowest activity of G6PD are destroyed while younger RBCs resist oxidant challenges [68]. Indeed, as RBCs age *in vivo* G6PD activity decreases [69] probably due to protein instability. This in turn promotes defects in antioxidant response and increased erythrophagocytosis. In agreement with other biochemical and metabolomics studies [70] -and in contrast with others [71] -the activity of G6PD in control RBCs decreased by 30% on the last day of storage compared to the activity *in vivo*, showing a defect in the GSH-dependent anti-oxidant systems in RBC after long storage. As previously noted, this might not necessarily correspond to compromised anti-oxidant capacity of these cells, as NADPH levels are mostly preserved during routine storage in SAGM [52,72]. However $\sim$50% activity loss was observed for G6PD activity in G6PD$^-$ RBC during the storage. This is consistent with carbonylation, metabolomics and proteomics data showing increased susceptibility to oxidation in G6PD$^-$ RBC and increased accumulation of oxidative lesion to key functional residues (*e.g.* cysteine β94 of hemoglobin beta) in long stored membrane and vesicles. Of note, cysteine β94 is critically implicated in maintaining nitrite homeostasis and redox status of Prdx2 thiols in stored RBCs [72].

In areas endemic for G6PD deficiency, a considerable percentage of G6PD$^-$ RBC units ($>$ 1.1% in Italy) [9] support transfusion therapy. The enrollment of G6PD deficient subjects as routine RBC donors has been questioned [3,73] and notably, the results from our *in vitro* model of transfusion are consistent with clinical reports. Indeed, lower 24 h post-transfusion recovery of stored RBCs from G6PD deficient donors when compared with normal RBCs was reported about fifty years ago [74]. Though transfusion of G6PD deficient RBCs is effective in correcting anemia, it is frequently accompanied by an immediate, mild post-transfusion hemolysis [10]. In fact, deleterious effects of G6PD$^-$ transfused blood appeared on neonates, children and patients with G6PD deficiency or those submitted to multiple transfusions compared to those reported on other adult patient groups [10,75]. However, other studies failed to reveal any deleterious effects of G6PD$^-$ blood transfusion to patients, especially to adult patients [76,77] and therefore blood donors are not routinely screened for G6PD deficiency, nor are there common guidelines regarding deferral of known G6PD$^-$ donors.

5. Conclusions

Our data report for the first time that the storability of the G6PD$^-$ RBCs might be similar or better compared to the G6PD$^-$ normal RBCs, at least in regard to certain storage lesion-related parameters like spheroechinocytosis. However, they further support concerns on the suitability of G6PD$^-$ subjects as routine donors. Experiments using an *in vitro* model of transfusion suggested that transfusion with G6PD deficient blood could result in increased hemolysis in certain conditions, such as in the case of recipients suffering from acute oxidative stress caused by infection or oxidative stress-inducing medications. On the other side, the chronic exposure of G6PD$^-$ subjects to oxidative stress might make them good recipients, as they better tolerate exposure to oxidatively damaged long-stored healthy RBCs and supernatants. Though the results shown here relate to grade II G6PD deficiency donors and non-hemolytic G6PD$^-$ "recipients", new studies will address whether the degree of G6PD deficiency or the favism affect the currently reported *in vitro* reactivity profiles. Currently ongoing clinical trials will contribute to clarify the actual clinical relevance of the observations we reported here. In this view, although the sample size used in this study is rather small for final conclusions to be drawn, we strongly believe that the *in vitro* results we describe here will inform the interpretation of the results from future large scale studies on the storability of G6PD deficient RBCs and their post-transfusion efficacy and effects.

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References

[1] P. Arese, A. De Flora, Pathophysiology of hemolysis in glucose-6-phosphate dehydrogenase deficiency, Semin. Hematol. 27 (1990) 1–40.
[2] G.F. Gaetani, A.M. Ferraris, M. Rolfo, R. Mangerini, S. Arena, H.N. Kirkman, Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocytes, Blood 87 (1996) 1595–1599.
[3] R.O. Francis, J.S. Jhang, H.P. Pham, E.A. Hod, J.C. Zimring, S.L. Spitalnik, Glucose-6-phosphate dehydrogenase deficiency in transfusion medicine: the unknown risks, Vox Sang. 105 (2013) 271–282.
[4] E.T. Nkhoma, C. Poole, V. Vannappagari, S.A. Hall, E. Beutler, The global prevalence of glucose-6-phosphate dehydrogenase deficiency: a systematic review and meta-analysis, Blood Cells Mol. Dis. 42 (2009) 267–278.
[5] A. Minucci, K. Moradkhani, M.J. Hwang, C. Zuppi, B. Giardina, E. Capoluongo, Glucose-6-phosphate dehydrogenase (G6PD) mutations database: review of the "old" and update of the new mutations, Blood Cells Mol. Dis. 48 (2012) 154–165.
[6] Glucose-6-phosphate dehydrogenase deficiency, WHO Working Group, Bull World Health Organ 67, 1989, pp. 601–611.
[7] T. Kanias, J.P. Acker, Biopreservation of red blood cells–the struggle with hemoglobin oxidation, FEBS J. 277 (2010) 343–356.
[8] E. Beutler, G6PD deficiency, Blood 84 (1994) 3613–3636.
[9] D. Maffi, M.T. Pasquino, L. Mandarino, P. Tortora, G. Girelli, D. Meo, G. Grazzini, P. Caprari, Glucose-6-phosphate dehydrogenase deficiency in Italian blood donors: prevalence and molecular defect characterization, Vox Sang. 106 (2014) 227–233.
[10] O. Shalev, N. Manny, R. Sharon, Posttransfusional hemolysis in recipients of glucose-6-phosphate dehydrogenase-deficient erythrocytes, Vox Sang. 64 (1993) 94–98.
[11] Blood Donor Selection: Guidelines on Assessing Donor Suitability for Blood Donation, Geneva, World Health Organization, 2012.
[12] W. Xu, B. Westwood, C.S. Bartsocas, J.J. Malcorra-Azpiazu, K. Indrak, E. Beutler, Glucose-6 phosphate dehydrogenase mutations and haplotypes in various

ethnic groups, Blood 85 (1995) 257–263.
[13] V.L. Tzounakas, H.T. Georgatzakou, A.G. Kriebardis, E.G. Papageorgiou, K. E. Stamoulis, L.E. Foudoulaki-Paparizos, M.H. Antonelou, I.S. Papassideri, Uric acid variation among regular blood donors is indicative of red blood cell susceptibility to storage lesion markers: a new hypothesis tested, Transfusion 55 (2015) 2659–2671.
[14] M. Harboe, A method for determination of hemoglobin in plasma by near-ultraviolet spectrophotometry, Scand. J. Clin. Lab. Invest. 11 (1959) 66–70.
[15] J.S. Raval, J.H. Waters, A. Seltsam, E.A. Scharberg, E. Richter, A.R. Daly, M. V. Kameneva, M.H. Yazer, The use of the mechanical fragility test in evaluating sublethal RBC injury during storage, Vox Sang. 99 (2010) 325–331.
[16] M.H. Antonelou, V.L. Tzounakas, A.D. Velentzas, K.E. Stamoulis, A.G. Kriebardis, I.S. Papassideri, Effects of pre-storage leukoreduction on stored red blood cells signaling: a time-course evaluation from shape to proteome, J. Proteom. (76 Spec No) (2012) 220–238.
[17] W.H. Reinhart, S. Chien, Red cell rheology in stomatocyte-echinocyte transformation: roles of cell geometry and cell shape, Blood 67 (1986) 1110–1118.
[18] T.L. Berezina, S.B. Zaets, C. Morgan, C.R. Spillert, M. Kamiyama, Z. Spolarics, E. A. Deitch, G.W. Machiedo, Influence of storage on red blood cell rheological properties, J. Surg. Res. 102 (2002) 6–12.
[19] E. Beutler, C. West, K.G. Blume, The removal of leukocytes and platelets from whole blood, J. Lab. Clin. Med. 88 (1976) 328–333.
[20] A.G. Kriebardis, M.H. Antonelou, K.E. Stamoulis, E. Economou-Petersen, L. H. Margaritis, I.S. Papassideri, Membrane protein carbonylation in non-leukodepleted CPDA-preserved red blood cells, Blood Cells Mol. Dis. 36 (2006) 279–282.
[21] A. D'Alessandro, T. Nemkov, M. Kelher, F.B. West, R.K. Schwindt, A. Banerjee, E. E. Moore, C.C. Silliman, K.C. Hansen, Routine storage of red blood cell (RBC) units in additive solution-3: a comprehensive investigation of the RBC metabolome, Transfusion 55 (2015) 1155–1168.
[22] A. D'Alessandro, T. Nemkov, K.C. Hansen, Z.M. Szczepiorkowski, L.J. Dumont, Red blood cell storage in additive solution-7 preserves energy and redox metabolism: a metabolomics approach, Transfusion 55 (2015) 2955–2966.
[23] A.G. Kriebardis, M.H. Antonelou, K.E. Stamoulis, E. Economou-Petersen, L. H. Margaritis, I.S. Papassideri, RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components, Transfusion 48 (2008) 1943–1953.
[24] M. Dzieciatkowska, C.C. Silliman, E.E. Moore, M.R. Kelher, A. Banerjee, K. J. Land, M. Ellison, F.B. West, D.R. Ambruso, K.C. Hansen, Proteomic analysis of the supernatant of red blood cell units: the effects of storage and leucoreduction, Vox Sang. 105 (2013) 210–218.
[25] T.J. van't Erve, C.M. Doskey, B.A. Wagner, J.R. Hess, B.W. Darbro, K.K. Ryckman, J.C. Murray, T.J. Raife, G.R. Buettner, Heritability of glutathione and related metabolites in stored red blood cells, Free Radic. Biol. Med. 76 (2014) 107–113.
[26] J.D. Roback, Vascular effects of the red blood cell storage lesion, Hematol. Am. Soc. Hematol. Educ. Progr. 2011 (2011) 475–479.
[27] V.L. Tzounakas, A.G. Kriebardis, H.T. Georgatzakou, L.E. Foudoulaki-Paparizos, M. Dzieciatkowska, M.J. Wither, T. Nemkov, K.C. Hansen, I.S. Papassideri, A. D'Alessandro, M.H. Antonelou, Data supporting that several physiological parameters of stored red blood cells are similar in glucose 6-phosphate dehydrogenase deficient and sufficient donors, Data In Brief, 2016.
[28] F. Gevi, A. D'Alessandro, S. Rinalducci, L. Zolla, Alterations of red blood cell metabolome during cold liquid storage of erythrocyte concentrates in CPD-SAGM, J. Proteom. (76 Spec No) (2012) 168–180.
[29] I. Messana, L. Ferroni, F. Misiti, G. Girelli, S. Pupella, M. Castagnola, B. Zappacosta, B. Giardina, Blood bank conditions and RBCs: the progressive loss of metabolic modulation, Transfusion 40 (2000) 353–360.
[30] T.A. Pertinhez, E. Casali, L. Lindner, A. Spisni, R. Baricchi, P. Berni, Biochemical assessment of red blood cells during storage by (1)H nuclear magnetic resonance spectroscopy. Identification of a biomarker of their level of protection against oxidative stress, Blood Transfus. 12 (2014) 548–556.
[31] A. Bordbar, P.I. Johansson, G. Paglia, S.J. Harrison, K. Wichuk, M. Magnusdottir, S. Valgeirsdottir, M. Gybel-Brask, S.R. Ostrowski, S. Palsson, O. Rolfsson, O. E. Sigurjonsson, M.B. Hansen, S. Gudmundsson, B.O. Palsson, Identified metabolic signature for assessing red blood cell unit quality is associated with endothelial damage markers and clinical outcomes, Transfusion 56 (2016) 852–862.
[32] A. D'Alessandro, K.C. Hansen, C.C. Silliman, E.E. Moore, M. Kelher, A. Banerjee, Metabolomics of AS-5 RBC supernatants following routine storage, Vox Sang. 108 (2015) 131–140.
[33] T. Nemkov, K.C. Hansen, L.J. Dumont, A. D'Alessandro, Metabolomics in transfusion medicine, Transfusion 56 (2015) 980–993.
[34] A.R. Tarlov, G.J. Brewer, P.E. Carson, A.S. Alving, Primaquine sensitivity. Glucose-6-phosphate dehydrogenase deficiency: an inborn error of metabolism of medical and biological significance, Arch. Intern. Med. 109 (1962) 209–234.
[35] F.C. Cheah, A.V. Peskin, F.L. Wong, A. Ithnin, A. Othman, C.C. Winterbourn, Increased basal oxidation of peroxiredoxin 2 and limited peroxiredoxin recycling in glucose-6-phosphate dehydrogenase-deficient erythrocytes from newborn infants, FASEB J. 28 (2014) 3205–3210.
[36] G.J. Bosman, Survival of red blood cells after transfusion: processes and consequences, Front. Physiol. 4 (2013) 376.
[37] J.M. Werre, F.L. Willekens, F.H. Bosch, L.D. de Haans, S.G. van der Vegt, A.G. van den Bos, G.J. Bosman, The red cell revisited–matters of life and death, Cell. Mol. Biol. 50 (2004) 139–145.
[38] S.C. Gifford, J. Derganc, S.S. Shevkoplyas, T. Yoshida, M.W. Bitensky, A detailed study of time-dependent changes in human red blood cells: from reticulocyte maturation to erythrocyte senescence, Br. J. Haematol. 135 (2006) 395–404.
[39] O. Castro, Viability and function of stored sickle erythrocytes, Transfusion 20 (1980) 695–703.
[40] R.L. Sparrow, G. Healey, K.A. Patton, M.F. Veale, Red blood cell age determines the impact of storage and leukocyte burden on cell adhesion molecules, glycophorin A and the release of annexin V, Transfus. Apher. Sci. 34 (2006) 15–23.
[41] W.W. Tuo, D. Wang, W.J. Liang, Y.X. Huang, How cell number and cellular properties of blood-banked red blood cells of different cell ages decline during storage, PLoS One 9 (2014) e105692.
[42] R.D. Propper, L.N. Button, D.G. Nathan, New approaches to the transfusion management of thalassemia, Blood 55 (1980) 55–60.
[43] T.E. Keegan, A. Heaton, S. Holme, M. Owens, E.J. Nelson, Improved post-transfusion quality of density separated AS-3 red cells after extended storage, Br. J. Haematol. 82 (1992) 114–121.
[44] T. Spanos, V. Ladis, F. Palamidou, I. Papassotiriou, A. Banagi, E. Premetis, C. Kattamis, The impact of neocyte transfusion in the management of thalassaemia, Vox Sang. 70 (1996) 217–223.
[45] P. Pisciotto, T. Kiraly, L. Paradis, R.M. Kakaiya, L. Rink, H.A. Pearson, Clinical trial of young red blood cells prepared by apheresis, Ann. Clin. Lab. Sci. 16 (1986) 473–478.
[46] A. D'Alessandro, A.G. Kriebardis, S. Rinalducci, M.H. Antonelou, K.C. Hansen, I. S. Papassideri, L. Zolla, An update on red blood cell storage lesions, as gleaned through biochemistry and omics technologies, Transfusion 55 (2015) 205–219.
[47] W.H. Reinhart, S. Chien, Echinocyte-stomatocyte transformation and shape control of human red blood cells: morphological aspects, Am. J. Hematol. 24 (1987) 1–14.
[48] A. De Flora, U. Benatti, L. Guida, G. Forteleoni, T. Meloni, Favism: disordered erythrocyte calcium homeostasis, Blood 66 (1985) 294–297.
[49] D. Teti, M. Crupi, M. Busa, A. Valenti, S. Loddo, M. Mondello, L. Romano, Chemical and pathological oxidative influences on band 3 protein anion-exchanger, Cell Physiol. Biochem. 16 (2005) 77–86.
[50] G. Pompeo, M. Girasole, A. Cricenti, G. Boumis, A. Bellelli, S. Amiconi, Erythrocyte death in vitro induced by starvation in the absence of Ca(2+), Biochim. Biophys. Acta 2010 (1798) 1047–1055.
[51] R. van Wijk, W.W. van Solinge, The energy-less red blood cell is lost: erythrocyte enzyme abnormalities of glycolysis, Blood 106 (2005) 4034–4042.
[52] A. D'Alessandro, G.M. D'Amici, S. Vaglio, L. Zolla, Time-course investigation of SAGM-stored leukocyte-filtered red bood cell concentrates: from metabolism to proteomics, Haematologica 97 (2012) 107–115.
[53] N.A. Lachant, N.A. Noble, B.A. Myrhe, K.R. Tanaka, Antioxidant metabolism during blood storage and its relationship to posttransfusion red cell survival, Am. J. Hematol. 17 (1984) 237–249.
[54] A. D'Alessandro, M. Dzieciatkowska, R.C. Hill, K.C. Hansen, Supernatant protein biomarkers of red blood cell storage hemolysis as determined through an absolute quantification proteomics technology, Transfusion (2016) (in press) http://dx.doi.org/10.1111/trf.13483. [Epub ahead of print].
[55] F.A. Kuypers, K. de Jong, The role of phosphatidylserine in recognition and removal of erythrocytes, Cell. Mol. Biol. 50 (2004) 147–158.
[56] C. Closse, J. Dachary-Prigent, M.R. Boisseau, Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium, Br. J. Haematol. 107 (1999) 300–302.
[57] D. Mittag, A. Sran, K.S. Chan, M.P. Boland, E. Bandala-Sanchez, O. Huet, W. Xu, R.L. Sparrow, Stored red blood cell susceptibility to in vitro transfusion-associated stress conditions is higher after longer storage and increased by storage in saline-adenine-glucose-mannitol compared to AS-1, Transfusion 55 (2015) 2197–2206.
[58] J.C. Zimring, S.L. Spitalnik, On the appropriate use and interpretation of animal models in transfusion medicine research, Transfusion 53 (2013) 2334–2339.
[59] R.M. Johnson, Y. Ravindranath, M.S. ElAlfy, G. Goyette Jr., Oxidant damage to erythrocyte membrane in glucose-6-phosphate dehydrogenase deficiency: correlation with in vivo reduced glutathione concentration and membrane protein oxidation, Blood 83 (1994) 1117–1123.
[60] N. Gurbuz, O. Yalcin, T.A. Aksu, O.K. Baskurt, The relationship between the enzyme activity, lipid peroxidation and red blood cells deformability in hemizygous and heterozygous glucose-6-phosphate dehydrogenase deficient individuals, Clin. Hemorheol. Microcirc. 31 (2004) 235–242.
[61] D. Mendez, M. Linares, A. Diez, A. Puyet, J.M. Bautista, Stress response and cytoskeletal proteins involved in erythrocyte membrane remodeling upon Plasmodium falciparum invasion are differentially carbonylated in G6PD A-deficiency, Free Radic. Biol. Med. 50 (2011) 1305–1313.
[62] K.S. Lang, B. Roll, S. Myssina, M. Schittenhelm, H.G. Scheel-Walter, L. Kanz, J. Fritz, F. Lang, S.M. Huber, T. Wieder, Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency, Cell. Physiol. Biochem. 12 (2002) 365–372.
[63] L. Bernini, B. Latte, M. Siniscalco, S. Piomelli, U. Spada, M. Adinolfi, P. L. Mollison, Survival of 51 Cr-labelled red cells in subjects with Thalassaemia-trait or G6pd deficiency or both abnormalities, Br. J. Haematol. 10 (1964) 171–180.
[64] F.E. Boas, L. Forman, E. Beutler, Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia, Proc. Natl. Acad. Sci. USA 95 (1998) 3077–3081.
[65] C.C. Silliman, D.R. Ambruso, L.K. Boshkov, Transfusion-related acute lung injury, Blood 105 (2005) 2266–2273.
[66] A. Kriebardis, M. Antonelou, K. Stamoulis, I. Papassideri, Cell-derived microparticles in stored blood products: innocent-bystanders or effective mediators of post-transfusion reactions? Blood Transfus. 10 (Suppl. 2) (2012) S25–S38.

[67] J.N. Harr, E.E. Moore, T.L. Chin, A. Ghasabyan, E. Gonzalez, M.V. Wohlauer, A. Banerjee, C.C. Silliman, A. Sauaia, Platelets are dominant contributors to hypercoagulability after injury, J. Trauma Acute Care Surg. 74 (2013) 756–762, discussion 762–755.
[68] P. Arese, V. Gallo, A. Pantaleo, F. Turrini, Life and death of glucose-6-phosphate dehydrogenase (G6PD) deficient erythrocytes - role of redox stress and band 3 modifications, Transfus. Med. Hemother. 39 (2012) 328–334.
[69] M.L. Brajovich, A. Rucci, I.L. Acosta, C. Cotorruelo, S. Garcia Borras, L. Racca, C. Biondi, A. Racca, Effects of aging on antioxidant response and phagocytosis in senescent erythrocytes, Immunol. Invest. 38 (2009) 551–559.
[70] D.K. Korgun, S. Bilmen, A. Yesilkaya, Alterations in the erythrocyte antioxidant system of blood stored in blood bags, Res. Commun. Mol. Pathol. Pharmacol. 109 (2001) 357–363.
[71] R.O. Francis, J. Jhang, J.E. Hendrickson, J.C. Zimring, E.A. Hod, S.L. Spitalnik, Frequency of glucose-6-phosphate dehydrogenase-deficient red blood cell units in a metropolitan transfusion service, Transfusion 53 (2013) 606–611.
[72] V.M. Harper, J.Y. Oh, R. Stapley, M.B. Marques, L. Wilson, S. Barnes, C.W. Sun, T. Townes, R.P. Patel, Peroxiredoxin-2 recycling is inhibited during erythrocyte storage, Antioxid. Redox Signal. 22 (2015) 294–307.
[73] A.M. Renzaho, E. Husser, M. Polonsky, Should blood donors be routinely screened for glucose-6-phosphate dehydrogenase deficiency? A systematic review of clinical studies focusing on patients transfused with glucose-6-phosphate dehydrogenase-deficient red cells, Transfus. Med. Rev. 28 (2014) 7–17.
[74] A.R. Orlina, A.M. Josephson, B.J. McDonald, The poststorage viability of glucose-6-phosphate dehydrogenase-deficient erythrocytes, J. Lab. Clin. Med. 75 (1970) 930–936.
[75] S.H. Nabavizadeh, A. Anushiravani, The prevalence of G6PD deficiency in blood transfusion recipients, Hematology 12 (2007) 85–88.
[76] F. Emamghorashi, F. Hooshmand, A.R. Mohtashamifar, Screening for glucose-6-phosphate dehydrogenase deficiency in blood donors, Transfusion 49 (2009) 2516.
[77] C.S. Huang, Y.C. Sung, M.J. Huang, C.S. Yang, W.S. Shei, T.K. Tang, Content of reduced glutathione and consequences in recipients of glucose-6-phosphate dehydrogenase deficient red blood cells, Am. J. Hematol. 57 (1998) 187–192.