Recipient's effects on stored red blood cell performance: the case of uremic plasma

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BACKGROUND: Despite universal administration of erythropoiesis-stimulating agents, patients with end-stage renal disease (ESRD) are at high risk for presenting persistent anemia. Due to ambiguities in optimal hemoglobin targets and evidence of recombinant human erythropoietin (EPO)-related toxicity, an increase in blood transfusions has been observed in chronic renal disease over the past years. The probable effects of uremic plasma on the performance of stored red blood cells (RBCs) after transfusion have not been investigated.

STUDY DESIGN AND METHODS: Leukoreduced RBCs after short or long storage in CPD-SAGM (n = 5) were assessed for hemolysis, surface removal signaling, reactive oxygen species (ROS) accumulation, and shape distortions before and after reconstitution with healthy (n = 10) or uremic plasma from ESRD patients (n = 20) for 24 hours at physiologic temperature, by using a previously reported in vitro model of transfusion.

RESULTS: Temperature and cell environment shifts from blood bag to plasma independently and in synergy affected the RBC physiology. Outcome measures at transfusion-simulating conditions might not be analogous to timing of storage lesion. The uremic plasma ameliorated the susceptibility of stored RBCs to hemolysis, phosphatidylserine externalization, and ROS generation after stimulation by oxidants, but negatively affected shape homeostasis versus healthy plasma. Creatinine, uric acid, and EPO levels had correlations with the performance of stored RBCs in ESRD plasma.

CONCLUSION: Renal insufficiency and EPO supplementation likely affect the recovery of donor RBCs and the reactivity of RBCs after transfusion by exerting both toxic and cytoprotective influences on them. ESRD patients constitute a specific recipient group that deserves further examination.

Prevalence of anemia in patients with advanced chronic renal insufficiency, including end-stage renal disease (ESRD), on regular hemodialysis (HD) treatment is high.[1] Clinical therapy with erythropoiesis-stimulating agents (ESAs), such as recombinant human erythropoietin (rHuEPO), has in many cases eliminated the need for blood transfusions. However, optimal hemoglobin (Hb) targets for rHuEPO therapy remain unclear. Moreover, a number of large clinical trials that highlighted some severe adverse effects of ESAs ("ESA toxicity"), along with changes in the regulatory environment that dictated a more conservative approach to ESA therapy, have led to an increase in blood transfusion rate in patients with renal disease during the past decade.[2] Similar to the general

ABBREVIATIONS: ESA(s) = erythropoiesis-stimulating agent(s); ESRD = end-stage renal disease; HD = hemodialysis; iROS = induced reactive oxygen species; PS = phosphatidylserine; ROS = reactive oxygen species tBHP = *tert*-butyl hydroperoxide; UA = uric acid.

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population, blood transfusion remains an effective therapeutic alternative to treat severe anemia and support surgery in chronic kidney disease patients, especially when a future renal transplantation is not a consideration.

The net outcome of transfusion therapy is dictated by the functional crosstalk between donor[3,4] and recipient genetics and other variables present in distinct patient groups suffering by acute or chronic diseases.[5] A hidden piece of the whole storage lesion picture becomes evident only after transfusion, when the stored RBCs meet other types or other level of stress.[6,7] And still, there is no evidence to exclude the possibility that some patients may better "host" the transfused RBCs by smoothing away their storage injuries in vivo, compared to the average recipient. Thus, the aim of this preliminary study was to evaluate the potential effects of variable host plasma composition on the performance of donor RBCs by using a previously reported[8] in vitro model of transfusion. Based on 1) the absence of any laboratory or clinical data on the translatability of RBC storage lesion into recovery and transfusion outcomes in patients with ESRD (who are at high risk for developing anemia) and 2) the previously established beneficial effects of plasma antioxidant capacity (a significant part of which is uric acid [UA]-dependent) on stored RBC performance,[9] uremic plasma that is commonly characterized by higher-than-average control levels of UA was studied by priority.

MATERIALS AND METHODS

Blood processing and study planning

Fifteen regular blood donors and 20 nondiabetic patients with ESRD under conventional HD treatment (thrice a week) with highly biocompatible filters were recruited in this study. Eight of them showed poor responsiveness to standard rHuEPO dose (ESA hyporesponders), and thus, they were treated with higher dose of rHuEPO to maintain the target Hb concentration (11 g/dL). All patients were receiving food supplements (carnitine 6 g/week, B1 100 mg/week, B6 100 mg/week, and B12 1 mg/week) and heparin (2800 ± 1000 IU/session) and were clinically stable at time of investigation. Subjects with uncontrolled hypertension, active infections, malignancy, inflammatory and autoimmune diseases, or blood transfusion over the past 3 months were excluded. Fresh blood from healthy subjects (including regular blood donors) and ESRD patients (before HD session) was collected into citrate or EDTA vacutainers. RBCs of five healthy blood donors were prepared from whole blood donation in CPD-SAGM units after prestorage leukoreduction (Haemonetics) and stored for 42 days at 4°C. The study has been submitted and approved by the Research Bioethics and BioSecure Committee of the Department of Biology/NKUA. Investigations were carried out in accordance with the principles of the Declaration of Helsinki. All blood donors and patients signed an informed consent before participating in this study.

In vitro model of transfusion

An in vitro model of transfusion[8] was used to stimulate the effects of uremic plasma on stored RBCs after transfusion in ESRD patients. CPD-SAGM-stored RBCs (n = 5) after short (for 8-10 days) or long (for 38-42 days) storage were reconstituted with either control (n = 10) or uremic plasma (n = 20; collected by centrifugation of fresh blood at 2500 × *g* for 15 min at 4°C) at 32% to 35% hematocrit. After incubation in 5% CO_2 atmosphere for 24 hours at 37°C under gentle agitation in a platform rocker, the cell suspensions were evaluated for hemolysis,[10] RBC morphology (by scanning electron microscopy [Philips SEM515] and blind assessment of at least 2000 cells from randomly chosen fields per sample), and intracellular accumulation of reactive oxygen species (ROS) with/without stimulation by oxidants (100 μmol/L *tert*-butyl hydroperoxide [tBHP] or 2 mmol/L diamide for 20 min at 37°C) by fluorometry (5-(and-6) chloromethyl-2′,7′-dichloro-dihydro-fluorescein diacetate acetyl ester labeling, Invitrogen, Molecular Probes).[11] Multicolor flow cytometry was used for the estimation of phosphatidylserine (PS) exposure by phycoerythrin-conjugated annexin V and fluorescein isothiocyanate–conjugated anti-CD235 (Clone GA-R2, HIR2; BD Pharmingen), as previously described.[8] Enumeration of circulating PS-positive microvesicles was performed by flow cytometry in citrated ESRD plasma placed in TruCount tubes.[12] Aliquots of RBCs at 4°C were tested under the same conditions (as internal controls used for normalization of the measurements).

Statistical analysis

All experiments were performed in triplicate. For statistical analysis, computer software (Statistical Package for Social Sciences, Version 22.0 for Windows, IBM Corp.) was used. Intergroup differences were evaluated by t test, after normalization to internal controls. Pearson's and Spearman's tests were performed to assess correlation (r) between parameters with/without normal distribution profiles, respectively. Significance was accepted at a p value of less than 0.05.

RESULTS

The uremic plasma ameliorates the susceptibility of stored RBCs to hemolysis, ROS generation, and surface removal signaling but negatively affects cell shape homeostasis versus healthy plasma

The RBCs tested in the reconstitution experiments exhibited low (<0.8%) levels of end-of-storage hemolysis (free Hb, 46.6 ± 12.3 mg Hb/dL; Fig. 1A). In general, 24-hour incubation of RBCs at physiologic temperature had no effect on endogenous ROS levels but triggered hemolysis, PS externalization, and susceptibility to ROS generation after stimulation by oxidants (induced ROS, iROS, mainly in younger cells). In contrast, it ameliorated the shape distortions seen in blood bag (patterned bars vs. dashed normalization lines in Fig. 1). Replacement of

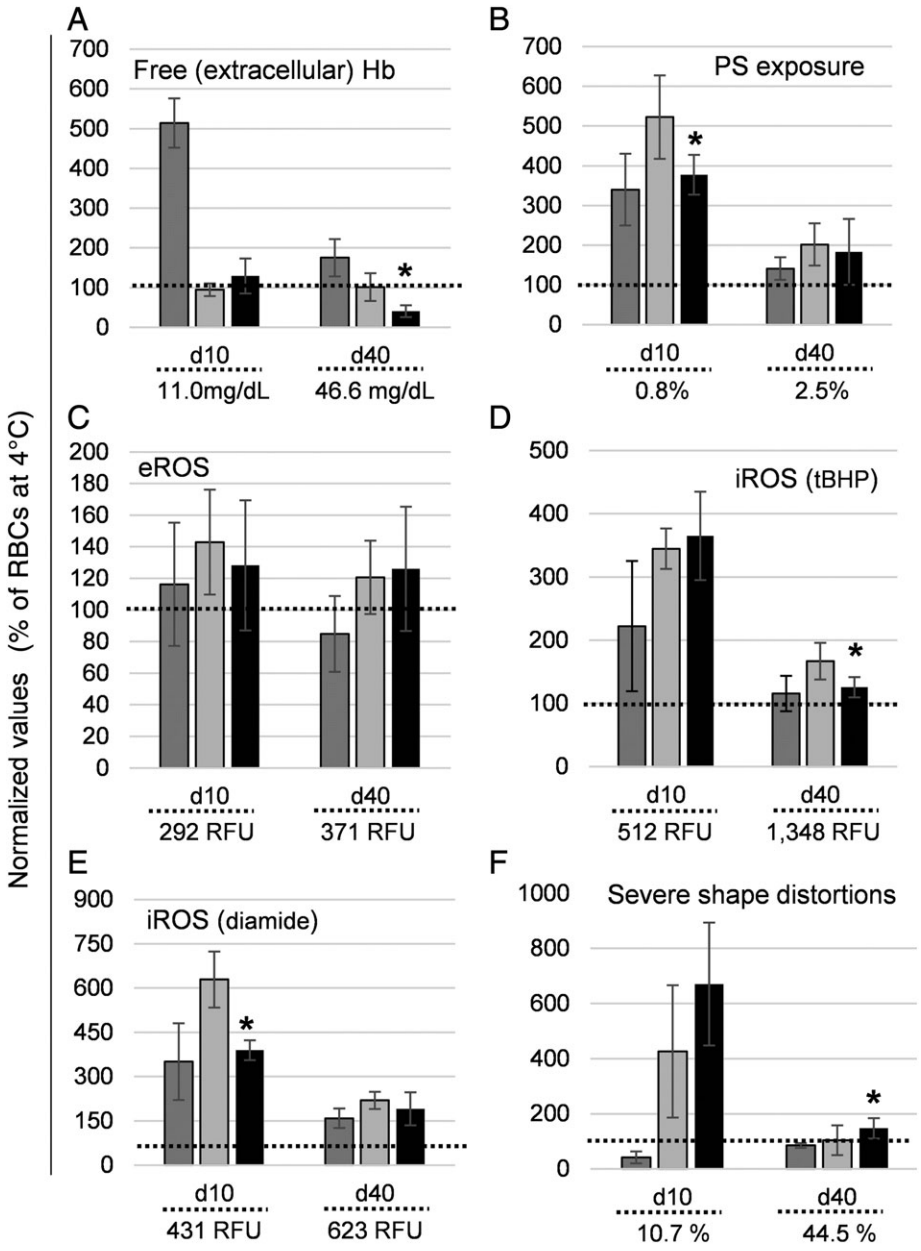

Fig. 1. Evaluation of RBCs (n = 5) stored for short (Day 10 [d10]) or for long (Day 40 [d40]) periods in CPD-SAGM for free Hb concentration (A), percentage of annexin V–positive RBCs (B), endogenous (eROS, C), or tBHP- (D) and diamide- (E) induced ROS accumulation (iROS) and percentage of severe shape distortions (F), before (whole RBCs) or after reconstitution of stored RBCs with ESRD (n = 20) or control (n = 10) plasma. Values were normalized to those of stored RBCs in situ at 4°C (100%, · · ·). The actual value for each normalization line per time point is shown on the x-axis labels. *p < 0.05 ESRD versus control plasma. Stored RBCs (24 hr/37°C): (■) supernatant; (■) control plasma; (■) ESRD plasma.

supernatant by healthy plasma in the reconstituted RBCs (to simulate the posttransfusion state) sharply decreased hemolysis (Fig. 1A) but exacerbated the shape homeostasis in younger stored RBCs (Figs. 1F and 2). Of note, PS exposure and responsiveness of reconstituted RBCs to oxidants were always higher than those measured in bag, throughout the storage period.

The biochemical composition of uremic plasma was typical of ESRD disease, showing higher urea (151 ± 29 mg/dL), creatinine (8.1 ± 2.0 mg/dL), UA (7.1 ± 2.1 mg/dL), ferritin (925 ± 469 ng/mL), and parathormone (256 ± 81 pg/mL) levels, as well as overaccumulation of total (40,000 ± 11,000 counts/μL) and RBC-derived (2,250 ± 800 counts/μL) annexin V–positive circulating microvesicles compared with

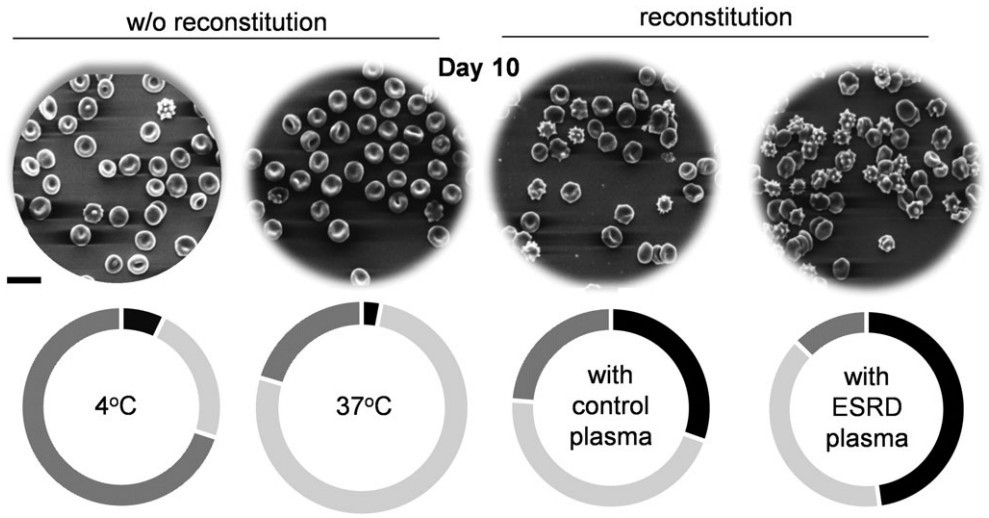

Fig. 2. Representative scanning electron micrographs (top) and shape distribution (bottom) of RBCs from regular blood donors after 10 days of storage in CPD-SAGM before or after reconstitution with ESRD or control plasma for 24 hours at 37°C (scale bar, 10 μm). (▒) Discocytes; (▓) mild shape distortions; (■) severe shape distortions.

healthy controls (20,500 ± 13,000 and 750 ± 500 counts/μL, respectively). Notwithstanding, it had an impressive antihemolytic effect on long-stored RBCs (Fig. 1A) and kept PS exposure on younger RBCs to lower levels compared to control plasma (Fig. 1B). Moreover, while there was no variation in endogenous ROS accumulation (Fig. 1C), ESRD plasma ameliorated the generation of ROS by old tBHP-stimulated stored RBCs (Fig. 1D) and by young diamide-stimulated stored RBCs (Fig. 1E). In stark contrast to those variables, the uremic plasma aggravated the shape heterogeneity of old RBCs compared to both control plasma and basal levels of RBCs (Figs. 1F and 2).

Creatinine, UA, and EPO levels are associated with the performance of stored RBCs

The high patient-to-patient variability in the above-mentioned metrics prompted us to examine in closer the probable contribution of individual plasma components. Statistical analysis of the hematologic, plasma biochemical, and RBC physiologic variables revealed significant inverse correlations between serum UA levels with the intracellular (endogenous and induced) ROS accumulation and the incidence of severe shape distortions in reconstituted RBCs (Figs. 3A-3D). In contrast to UA, serum creatinine had positive correlation with PS exposure levels on younger stored RBCs ($r = 0.662$, $p = 0.019$; Fig. 3E). Shape abnormalities had further correlations with both annexin V positivity and induced ROS (iROS; Fig. 3F,G), that further correlated with extracellular (free) Hb levels (Fig. 3H).

Finally, the plasma of ESRD patients showing poor responsiveness to rHuEPO therapy, and thus receiving high dose of it (21,200 ± 5020 IU/week) was more effective in ameliorating hemolysis, PS exposure, and iROS levels in old-stored RBCs compared to both the plasma of responsive ESRD patients (receiving doses of 8400 ± 5683 IU/week) (Fig. 3I) and the plasma of controls. No interpatient differences in the percentage of shape distortions were observed (data not shown).

DISCUSSION

This study evaluated whether recipient variation in plasma biochemicals and, specifically, the typical uremic plasma, might affect the performance of stored RBCs in an in vitro model of transfusion.

Two sides of the storage lesion: RBCs in CPD-SAGM versus healthy plasma

Red blood cell transfusion involves transition of RBCs from preservative medium at 2 to 6°C to plasma at physiological temperature (37°C). According to this study, temperature shift per se triggered hemolysis and surface removal signaling in a storage time–dependent manner but tended to normalize RBC shape pathologies. The healthy plasma blunted hemolysis of stored RBCs but not the PS-triggering effect of high temperature, and moreover, it aggravated the morphologic distortions. Thus, the physiologic characteristics of RBCs in blood bag and the well-characterized timing of RBC storage lesion (e.g., shape distribution at early storage) may or may not be quantitatively analogous to those detected in an in vivo-simulating environment. For instance, while the susceptibility of reconstituted young or old RBCs to hemolysis paralleled that of stored RBCs, the susceptibilities of younger RBCs to PS exposure, ROS generation, and severe shape distortions were substantially disproportionate to the baseline levels and clearly higher compared to those of older cells in blood bag. These data are consistent with

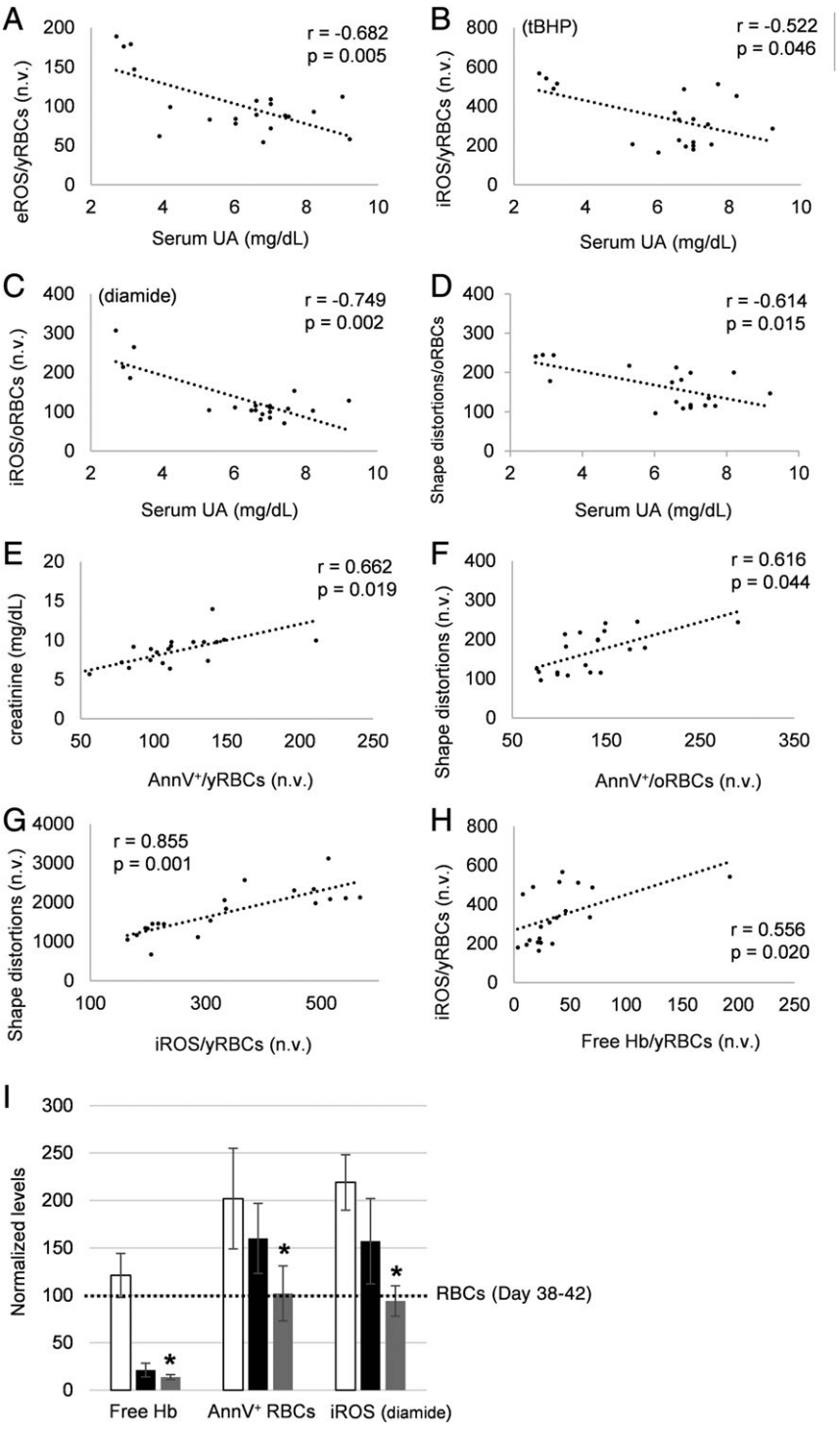

Fig. 3. (A-H) Scatterplots showing significant ($p < 0.05$) correlations between uremic plasma (UA, creatinine, free Hb) and donor RBC (annexin V [AnnV+] positivity, eROS, iROS, severe shape distortions) factors in the reconstituted cell suspensions. Apart from UA and creatinine, the values were normalized to those of RBCs at 4°C. y/o = young- (Days 8-10)/old- (Days 38-42) stored RBCs, respectively. (I) Significant differences in the physiologic characteristics of RBCs (n = 5) stored for 38 to 42 days in CPD-SAGM after reconstitution with control (□; n = 10) or uremic plasma from ESRD patients responsive (■; n = 12) or hyporesponsive to rHuEPO (■ n = 8). *$p < 0.05$ responsive versus hyporesponsive patients. Values were normalized to those of stored RBCs at 4°C (100%, dashed line).

those of other studies showing that the RBCs are impacted early enough during storage by changes in their physiologic environment.[13] Moreover, PS exposure on reconstituted RBCs of Day 40 was not significantly higher compared to that seen on Day 10 RBCs, despite the fact that the percentage of PS-positive RBCs differed significantly between the early and late periods of storage. The individual contribution of each "neo-modification" on RBC recovery after transfusion apparently depends on the activity of clearance mechanisms (extravascular hemolysis) and other physiologic conditions (e.g., status of vascular endothelium) that were not evaluated by this in vitro model of transfusion.

ESRD patients constitute a nonaveraged and heterogeneous RBC recipient group

The unique composition of uremic plasma seemed to improve the performance of young or old RBCs in the physiologic environment, at least with respect to hemolysis, surface signaling, and iROS production over healthy plasma. The antihemolytic activity of uremic plasma was previously observed in fresh (nonstored) RBCs;[12] however, its effect on Day 40 RBCs was impressive when compared with the baseline levels. This observation suggests better recovery of stored RBCs in ESRD than in healthy recipients.

Pleiotropic cytoprotective effects of EPO have been reported on mature RBCs, regarding antioxidant defenses, PS surface signaling,[14] and senescence.[15] Either through a presumptive direct contact with RBCs[15,16] or as an effective hydroxyl radical scavenger[17] EPO might alleviate the oxidative stress, resulting in lower susceptibility to hemolysis and erythrophagocytosis.[18] While previous investigations have shown beneficial effects of EPO on ESRD RBCs,[12,19] the current study reports that EPO-related cytoprotection (revealed after comparison of plasma of rHuEPO responders and hyporesponders) concerns heterologous RBCs as well. As EPO hyporesponsiveness has been associated with an increased incidence of blood transfusions in ESRD,[20] further examination of these findings in large cohorts of patients could change the decision-making process in transfusion therapy. Apart from the antioxidant effects, up regulation of nitric oxide production by RBCs and endothelium is a further cytoprotective aspect of EPO, especially useful in transfusion settings.[21]

The high antioxidant capacity of uremic plasma, which is mainly UA-based,[12] may further benefit the recovery of donor RBCs. Serum UA levels that are positively correlated with the RBC life span in chronic HD patients[22] affect storage lesion metrics in RBCs under several storage strategies.[9,13,23] This study further extends the influential central position of UA in the transfusion context by including a recipient-related aspect. The tBHP oxidant produces radicals, and thus, a UA-enriched plasma may effectively quench in part the iROS levels in old-stored RBCs. As for diamide, which targets cysteine or thiol residues, its effect decreased at the end of the storage due to the increased storage-dependent oxidation of cysteine residues. However, in contrast to the beneficial effect of donor-related UA on storage spheroechinocytosis,[9,23] the high-UA uremic plasma was less effective in lowering shape heterogeneity of stored RBCs compared to healthy plasma, despite the fact that the UA levels of the uremic plasma negative correlated with severe shape distortions in old-stored RBCs (Fig. 3D). This is probably attributed to the higher heterogeneity of uremic plasma compared to that of preservative medium. Apart from UA, creatinine (Fig. 3E) and probably other uremic toxins with inflammatory, osmotic, or exovesiculation-triggering reactivities, as well as certain comorbidities, may also distort shape homeostasis in old stored RBCs. Irreversible morphologic damages (that reflect a systemic inflammatory profile in many pathologies) render RBCs susceptible to clearance by phagocytosis after transfusion and, thus, may decrease oxygen unloading to the microcirculation of ESRD patients. Of note, the permeabilities of RBC membrane to creatinine and UA, which are dependent on both temperature and other plasma components, differ between uremic and healthy RBCs.[24] It is interesting to note that despite its antioxidant activity, EPO counteracts only in part PS translocation in RBCs, due to its additional effect upon RBC calcium levels, potentially leading to changes in cell shape and volume.[14] Probably for this reason increased RBC PS exposure is a hallmark of chronic renal failure.[12,25] PS exposure and shape distortions in reconstituted stored RBCs might be associated with their exovesiculation profile once inside the uremic environment, but this assumption needs further study by different experimental approaches.

In conclusion, ESRD patients likely constitute a specific recipient group. Severity of the disease or therapy-associated factors, including degree of anemia, inflammation status, endothelium defects, activity of RBC clearance systems, concentration and composition of extracellular vesicles, and EPO/iron supplementation, may all affect the recovery of donor RBCs as well as the reactivity of RBCs after transfusion. The current study suggests that performance of stored RBCs in vivo may be not quantitatively analogous to the baseline damage after the transition of cells in a new and highly heterogenous environment. Increased understanding of the role that donor/recipient factors play in transfusion efficacy is indispensable to realizing the vision of personalized transfusion therapy.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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