Donor-specific individuality of red blood cell performance during storage is partly a function of serum uric acid levels

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BACKGROUND: Previous investigations in leukoreduced units of red blood cells (RBCs) in mannitol additive solution revealed the close association of uric acid (UA) levels in vivo with the susceptibility of RBCs to storage lesion markers. In this study, we examined whether UA has a similar correlation with the capability of RBCs to cope with the oxidative provocations of storage under different conditions, namely, in CPDA-1 and in the absence of leukoreduction.

STUDY DESIGN AND METHODS: The UA-dependent antioxidant capacity of the supernatant was measured in nonleukoreduced units of RBCs in CPDA (n = 47). The possible effect of UA variability on the storage lesion profile was assessed by monitoring several physiologic properties of RBCs and supernatant, including cell shape, reactive oxygen species, and size distribution of extracellular vesicles, in units exhibiting the lowest or highest levels of UA activity (n = 16) among donors, throughout the storage period.

RESULTS: In stored RBC units, the UA-dependent antioxidant activity of the supernatant declined as a function of storage duration but always in strong relation to the UA levels in fresh blood. Contrary to units of poor-UA activity, RBCs with the highest levels of UA activity exhibited better profile of calcium- and oxidative stress–driven modifications, including a significant decrease in the percentages of spherocytes and of 100- to 300-nm-sized vesicles, typically associated with the exovesiculation of stored RBCs.

CONCLUSION: The antioxidant activity of UA is associated with donor-specific differences in the performance of RBCs under storage in nonleukoreduced CPDA units.

During their preservation at blood banks, red blood cells (RBCs) undergo several physiological alterations and/or deteriorations collectively known as "RBC storage lesion." The mechanisms underlying the storage lesion remain uncertain; however, there is evidence that a significant part of it is driven by oxidative stress.[1] In fact, both reduction of antioxidant activity in stored RBCs and supernatant and oxidation of key lipid and protein components, including hemoglobin (Hb), have been reported during storage

ABBREVIATIONS: DLS = dynamic light scattering; EV(s) = extracellular vesicle(s); MDA = malondialdehyde; ROS = reactive oxygen species; UA = uric acid; UA-AC = UA-dependent antioxidant capacity.

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under various strategies and preservative solutions.[2] Of note, some critical aspects of the storage lesion, including the redox equilibrium and the decay of metabolism, are considered donor related. Lipid peroxidation[3] and reduced glutathione[4] content of RBCs, for instance, were shown to be donor specific, while the levels of clusterin and nitrogen species in stored RBC units were reported to be a function of their values in fresh, non-stored blood.[5]

In the same context, recent investigations of donor variation effect on RBC storage lesion in leukoreduced units of RBCs in mannitol additive solution (AS) pointed out a close association between the in vivo levels of the natural antioxidant uric acid (UA) and the susceptibility of RBCs to storage lesion markers.[6] Having in mind that UA on average represents as much as 60% of the total antioxidant capacity of the plasma,[7] in this study, we examined whether the donor-related levels of UA reveal, equally well, the intrinsic capability of RBCs to cope with the oxidative provocations of storage under conditions of shorter biopreservation capability, that are characterized by more severe oxidative lesions, namely, in the absence of AS and leukoreduction. Thus, the aim of this study was to provide evidence regarding the potential usefulness of UA as a donor-specific determinant or candidate biomarker of storage quality.

MATERIALS AND METHODS

Blood processing and study planning

Forty-seven male regular blood donors (18-25 years old) who met the established criteria for donation (e.g., Hb levels $>$ 13.5 g/dL) were recruited in this study. Fresh blood was collected into citrate-containing tubes, while RBC storability was evaluated in RBCs prepared in CPDA-1 bags, stored for 35 days at 4°C, in the absence of prestorage leukoreduction. For most assays, sampling was performed at the beginning, in the middle, and on the last day (Day 35) of the storage period. To test the dynamics of prestorage UA levels in discriminating the blood components under investigation, we performed a posteriori evaluation of two groups of RBCs that had been prepared from donors with quite different (Δ_{UA} = 2.3g/dL), yet normal, levels of plasma UA (Fig. 1A): the group of low-UA (4.2 $\pm$ 0.5g/dL, n = 8) versus that of high-UA (6.5 $\pm$ 0.3 g/dL, n = 8) units ($p < 0.01$). The study has been submitted and approved by the Research Bioethics and BioSecure Committee of the Faculty of Biology/NKUA. Investigations were carried out in accordance with the principles of the Declaration of Helsinki.

Hemolysis and UA-associated antioxidant capacity

In-bag hemolysis was calculated by using Drabkin's reagent (Sigma Aldrich),[8] as previously described.[6] Serum UA was measured using the automatic analyzer (Hitachi 902, Roche). Total antioxidant capacity and the UA-dependent antioxidant capacity (UA-AC) were determined in serum and supernatant samples, in the absence or presence of uricase (Sigma Aldrich) treatment, respectively,[9] by using the ferric reducing antioxidant power assay.[10]

Oxidative stress markers

Intracellular reactive oxygen species (ROS) accumulation in stored RBCs labeled with the redox-sensitive probe CM-H_2DCFDA (Invitrogen, Molecular Probes) was estimated by fluorometry.[5] Lipid peroxidation was assessed in the supernatant of RBC units by measuring the levels of malondialdehyde (MDA), a natural by-product of lipid peroxidation. After deproteinization of the supernatant with 15% trichloroacetic acid, thiobarbituric acid was added (all chemicals by Sigma Aldrich) and the absorption of the chromogenic MDA-thiobarbituric acid complex, produced after heating of the samples at 95°C for 50 minutes, was measured at 532 nm. Individual measurements were plotted against a standard curve of samples with known MDA concentration.

Intracellular calcium accumulation and scanning electron microscopy

Intracellular calcium accumulation in stored RBCs was fluorometrically estimated by using the calcium indicator Fluo-4AM (Invitrogen, Molecular Probes), as previously described.[6] Scanning electron microscopy was used for the assessment of RBC morphology. Briefly, after fixation and postfixation in 2% glutaraldehyde and 1% osmium tetroxide (Serva), respectively, RBCs were gradually dehydrated in ascending ethanol series. Dehydrated samples were coated with gold palladium before examination in a Philips SEM515 microscope.

Extracellular vesicles evaluation by dynamic light scattering

After collection of the supernatant (2000 $\times$ *g*, for 10 min at 4° C) and filtering through sterile 0.8-mm pore size syringe-driven nitrocellulose filter units (Millipore), the samples were ultracentrifuged at 30,000 $\times$ *g* for 1 hour at 4°C, conditions commonly used for the isolation of bigger-size extracellular vesicles (EVs), or "microvesicles," of 100 to 450 nm (although small EVs of 60-100 nm, typical "exosomes," coprecipitate). The pellet of EVs was resuspended in phosphate-buffered saline and the supernatant recentrifuged at 200,000 $\times$ *g* for 1 hour at 4°C for isolating the smaller EVs in the exosome pellet. For EV sizing, a high performance two angle particle and molecular size analyzer (Zetasizer Nano ZS, Malvern) was used. The instrument operated at a wavelength of 633 nm and was equipped with a helium-neon laser at the standard angle of 173° and a glass cuvette with square aperture. Dynamic

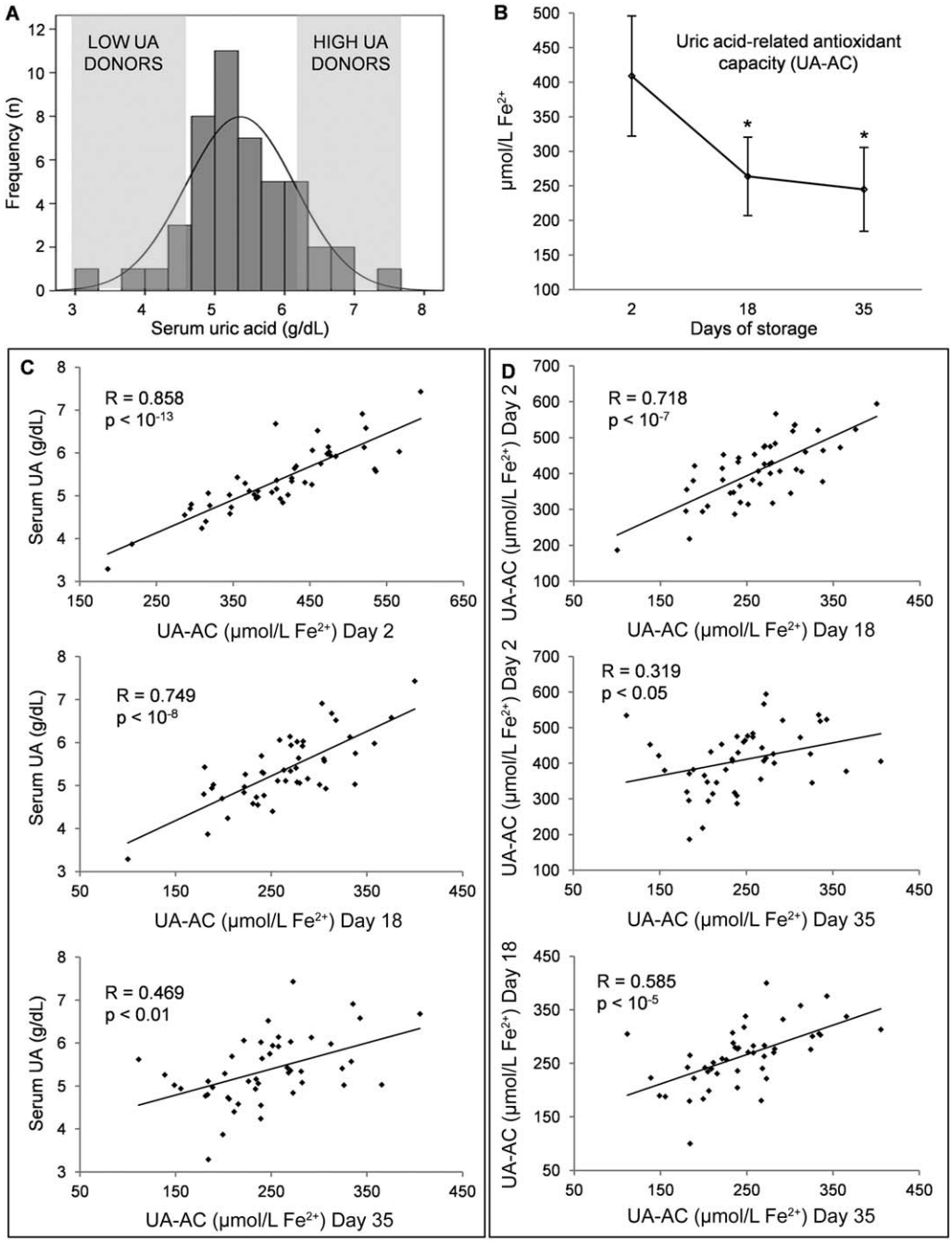

Fig. 1. Evaluation of donor's serum UA levels as a potential signature on stored RBCs. (A) Histogram showing the normal distribution of predonation serum UA levels among regular blood donors (n = 47). The two light gray areas indicate the donors comprising the high- (n = 8) and low- (n = 8) UA groups. (B) Time course evaluation (mean ± SD) of UA-AC during storage. *p < 0.05 storage versus Day 2. (C) Scatter plots demonstrating the strong and significant correlations between the UA levels in fresh blood and the UA-AC of RBCs stored for any storage period (n = 47). (D) Significant correlations between the UA-AC levels of RBC units throughout storage (n = 47).

light scattering (DLS) was adopted to evaluate the hydrodynamic diameter and the polydispersity of the EVs. The calculation of the hydrodynamic radius was based on the Einstein-Stokes equation:

$$R_H = (k_B T)/(6\pi\eta D)$$

where k_B is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, and D is the diffusion

coefficient. The vesicle solutions were prepared in a dilute concentration approximately equal to 0.1% (wt/wt). The DLS measurements showed monodispersed EV populations (polydispersity < 0.25).

Statistical analysis

For statistical analysis, computer software was used (SPSS, Version 22.0, IBM Corp.). All variables were tested for normal distribution using the Shapiro-Wilk test. Correlation between variables (Pearson and Spearman) and intergroup differences (t test and Mann-Whitney) were assessed according to the distribution profiles of each variable. Significance was accepted at a p value of less than 0.05.

RESULTS

The antioxidant activity of UA in the RBCs was proportional to the duration of storage and the donor- and unit-specific levels of UA concentration and activity

The units under investigation exhibited normal, acceptable (<0.8%) levels of end-of-storage hemolysis (0.259 ± 0.079%). The donors demonstrated normal range variation in UA levels in fresh blood (5.57 ± 1.25 g/dL), that revealed a typical Gauss distribution (Fig. 1A). The UA-AC of the supernatant declined gradually with storage duration and at significant levels versus Day 2 (Fig 1B). For all the RBC units (n = 47), the UA-AC of the supernatant at any time point of storage period was proportional to the baseline UA concentration in serum (Fig. 1C) and to the UA activity of the same unit at any earlier time point of the storage period (Fig. 1D). UA concentration in the serum and UA-AC in the supernatant were not correlated with the levels of in-bag hemolysis.

Some markers of oxidative stress were down-expressed in units prepared from high-UA donors

Lipid peroxidation of the supernatant steadily increased as a function of storage duration. A posteriori comparison of MDA levels in the supernatant of RBC units prepared from donors exhibiting either low or high levels of serum UA concentration (Δ_{UA} = 2.3g/dL between high- and low-UA group, n = 8 each) revealed statistically lower MDA concentration ($p < 0.05$) in the high-UA group at the early period of storage (Fig. 2A). Intracellular ROS were significantly lower ($p < 0.05$) in the high-UA units compared to those of the low-UA group, both at the middle and at the end of the storage period (no measurement was available for Day 2 samples; Fig. 2B). As expected, ROS accumulation increased with preservation time in all units, showing a notable, inverse correlation with serum UA concentration (n = 47, R = –0.453, $p < 0.01$ for Day 35 of storage).

Calcium accumulation, morphologic modifications, and EVs in the size range of 100 to 300 nm were also lower in the high-UA CPDA-1 units at the end of storage

Grouping of blood units according to the donor-related concentration of UA in the serum showed significantly different and inverse levels of intracellular calcium (Fig. 2C) and percentage of irreversibly modified RBCs, mostly spheroechinocytes (Fig. 2D, E), between the two groups, with better indexes in the units produced from high-UA donors (22.5 ± 2.9% vs. 27.1 ± 1.6% spheroechinocytes, high- vs. low-UA group, respectively).

To comparatively evaluate the size distribution of the EVs released by the stored cells in the supernatant of high- (n = 5) and low-UA (n = 5) units at the middle and the end of the storage period, we used DLS. Repeated DLS analyses of the 30,000 × *g* precipitates revealed a trend for more small EVs but less EVs of 100 to 300 nm, typical of the class of microvesicles, in the high-UA group compared to the low-UA group from the middle of the storage period that reached significance ($p < 0.05$) at the end of storage (Fig. 3A). Subsequent analyses of the 200,000 × *g* precipitates (exosomes) verified the above-mentioned trend for a different size distribution of EVs in the two groups, without, however, any statistical significance between them (Fig. 3B).

DISCUSSION

UA as a donor's "fingerprint" on RBCs

This study evaluated whether the donor-related variation in serum UA levels is associated with the storability of donated RBCs under storage in CPDA-1. Previous studies from our team in a smaller number of leukoreduced RBC units in CPD/SAGM have shown that several cellular and supernatant characteristics, including the UA-dependent antioxidant activity, fluctuate throughout the storage period in close relationship to their values in vivo.[5] In addition, Bardyn and colleagues[11] recently showed that variation in the extracellular antioxidant activity during storage follows the UA levels in the supernatant of leukoreduced CPD/SAGM units. Thus, we ought to find out whether the UA at baseline can reveal, with the same efficacy, some aspects of the RBC storability under alternative storage strategies characterized by different biopreservation features and levels of stressful stimuli, arising by the absence of ASs and prestorage leukoreduction.[12,13] Apart from the fact that similar strategies are routinely used in the European south and other countries worldwide, the choice of an alternative system would serve in revealing the dynamics of UA as a candidate storability "biomarker," in case of being storage medium and strategy independent.

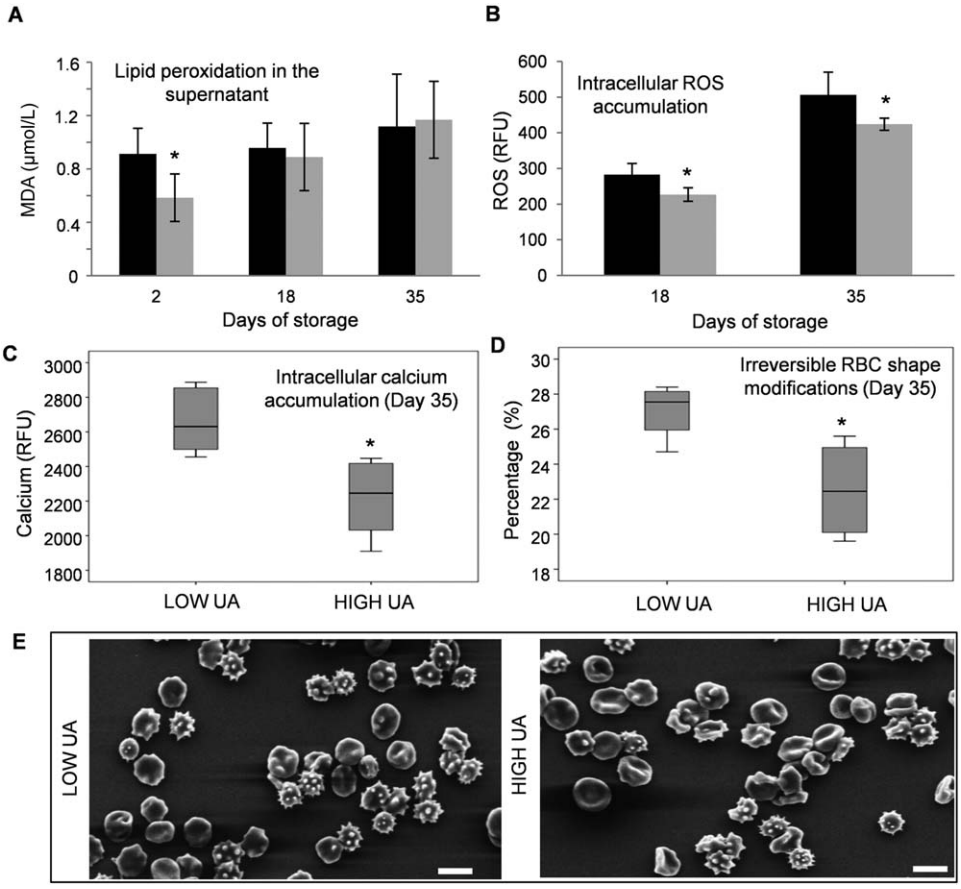

Fig. 2. Classification of CPDA-1 blood units in two groups (n = 8 each) according to the serum UA levels. (A) Lipid peroxidation of the supernatant and (B) intracellular ROS accumulation during storage. Data are mean ± SD. *p < 0.05 high (■) versus low (▒) UA. (C, D) Box plots showing the different levels of intracellular calcium accumulation and irreversibly modified RBCs, respectively, between the two groups on the last day of storage. *p < 0.05 high versus low UA. (E) Representative scanning electron micrographs of stored RBCs at the end of storage (scale bars = 10 μm).

Our study, by using a nonnegligible number of regular donors, showed that serum UA can disclose a significant part of the supernatant's antioxidant capacity that is clearly donor-related. Since not all variables were measured before and during storage, little can be said on whether the interdonor differences had a greater effect compared to the storage itself. Despite that, in all cases, the UA-AC decreased with storage by a rate that was linearly correlated to the baseline UA of the donor, although the respective correlation coefficients were also decreasing progressively with the storage duration. This fact suggests that the influence of serum UA on the supernatant UA-AC was time dependent, due to either UA metabolism in bag (to 5-hydroxyisourate and/or allantoin) and/or its influx into the stored RBCs. Moreover, the UA-AC levels of each unit at every time point of the storage period were strongly correlated with the values at any previous storage time, highlighting the universality of variation in UA-AC in RBCs. Finally, previous works on different storage systems,[5,6] along with works on young female donors (manuscript in preparation), who exhibit lower absolute extracellular antioxidant capacity compared to young male donors,[11] also suggested that the storage profile, at least in terms of UA-AC, clearly follows the interdonor differences in the prestorage levels. Consequently, along with other antioxidant variables, such as GSH[4] and RBC physiologic properties, such as the osmotic fragility,[14] this study suggests that UA-AC can be safely considered a donor-associated and in vivo measurable feature with the potential to reveal qualitative characteristics of the stored RBC units.

Serum UA classifies the RBCs according to their storability profile

Since the levels of serum UA demonstrated a normal distribution profile among donors, we used the "tails" of the Gaussian curve to study the potential impact of widely different baseline levels of UA on certain storability markers (namely, the potential existence of a UA threshold), as previously shown by a similar study in leukoreduced units of

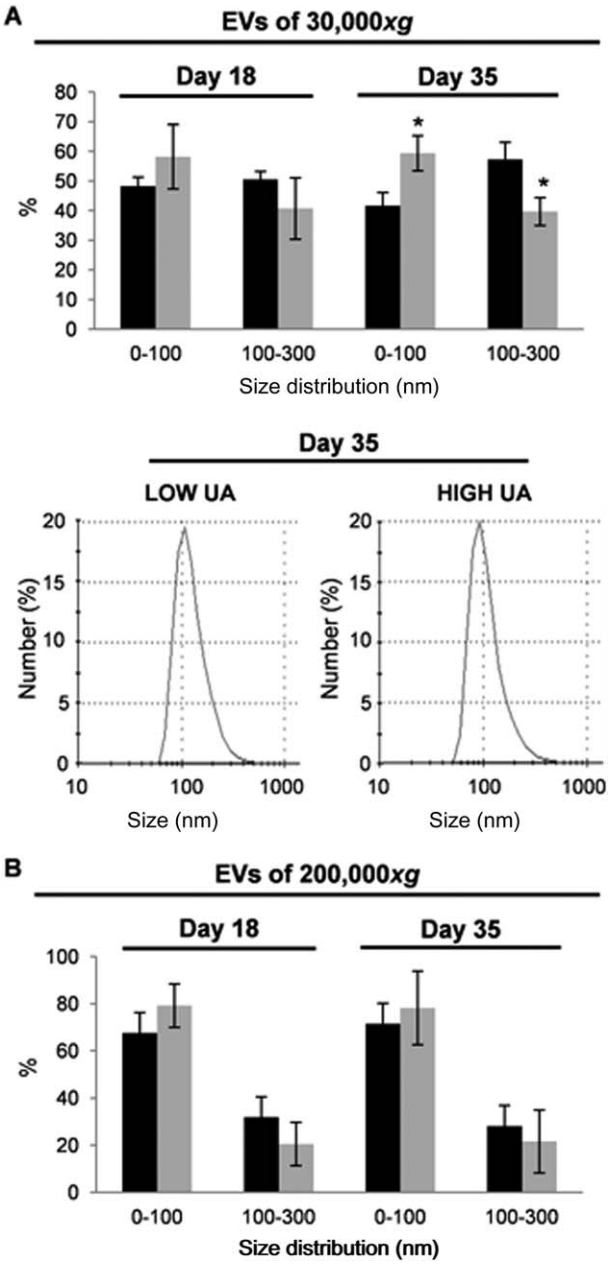

Fig. 3. Size distribution analysis of EVs by DLS (n = 5 for each group). (A) Size distribution of EVs (mean ± SD) after centrifugation of the supernatant at 30,000 × *g* in units from donors exhibiting low (■) or high (■) UA levels in vivo (top panel) and representative distribution curves (bottom panel). *p < 0.05 high versus low UA. (B) No difference was observed in the size of smaller vesicles collected after a second centrifugation of the supernatant at 200,000 × *g* (mean ± SD).

RBCs stored in CPD/SAGM.[6] Units from donors located at the upper sites of the curve (higher-UA donors) exhibited lower supernatant's lipid peroxidation at the beginning of the storage period and lower intracellular ROS accumulation after the middle of the storage period in CPDA-1, compared to the units of poor-UA donors. This profile might be related to the ability of UA to act both extra- and intracellularly. In fact, the UA-AC of the supernatant on Day 2 of storage was high enough to protect it from lipid peroxidation. However, as storage proceeded, the decreasing pH of the supernatant[15] may trigger the influx of UA into the stored RBCs, lowering thus the intracellular ROS levels.[16] Such a protective role of UA has been also shown in platelet (PLT) concentrates where addition of urate in excess leads to lower ROS production.[17] Moreover, the UA present in the uremic plasma exerts a strong antioxidant effect compared to healthy plasma on both healthy RBCs and RBCs from subjects with severe renal disease in vitro,[18] while its levels can effectively predict the RBC life span in chronic hemodialysis patients.[19]

Further end-of-storage diversification of the blood units, in favor of the high-UA ones, include the interrelated variables of intracellular calcium accumulation and severe shape modifications that can be also associated with a better antioxidant capacity.[20] Of note, those cells produce proportionally less EVs in the size range of 100 to 300 nm that are characteristic of the Hb-containing exovesicles released in nonleukoreduced CPDA-1 units (microvesicles/microparticles with mean sizes of 137-160 nm), as previously shown by immune-electron microscopy.[21] This comparative analysis verifies that oxidative stress represents one of the general mechanisms underlying cellular (including RBCs and PLTs) EV formation.[22] Since our samples were not leukoreduced, that pool of EVs contained the PLT-derived EVs as well, that are expected to be enriched in EVs in the size range of exosomes (<100 nm).

Recent studies have suggested that UA and the purine salvage metabolic pathway play a role in the progression of the RBC storage lesion.[6,23] In support of these findings, our results underline the importance of donor-related serum UA levels in respect to the antioxidant activity of RBCs and highlight the probable role of UA in the performance of RBCs under storage in nonleukoreduced CPDA-1 units. Whether UA variation is more or less important compared to other donor variables linked to RBC storability, remains to be clarified. Despite that, it is clearly associated with certain aspects of RBC storage lesion in a donor-dependent but storage medium– and strategy-independent way. Thus, UA as a donor's signature in blood components may indeed be a very promising candidate biomarker of RBC storage lesion.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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