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ORIGINAL PAPER

The antioxidant capacity of erythrocyte concentrates is increased during the first week of storage and correlated with the uric acid level

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Vox Sanguinis

Background and Objectives Red blood cells (RBCs) suffer from lesions during cold storage, depending in part on their ability to counterbalance oxidative stress by activating their antioxidant defence. The aim of this study was to monitor the antioxidant power (AOP) in erythrocyte concentrates (ECs) during cold storage.

Materials and Methods Six ECs were prepared in saline-adenine-glucose-mannitol (SAGM) additive solution and followed during 43 days. The AOP was quantified electrochemically using disposable electrode strips and compared with results obtained from a colorimetric assay. Haematological data, data on haemolysis and the extracellular concentration of uric acid were also recorded. Additionally, a kinetic model was developed to extract quantitative kinetic data on the AOP behaviour.

Results The AOP of total ECs and their extracellular samples attained a maximum after 1 week of storage prior to decaying and reaching a plateau, as shown by the electrochemical measurements. The observed trend was confirmed with a colorimetric assay. Uric acid had a major contribution to the extracellular AOP. Interestingly, the AOP and uric acid levels were linked to the sex of the donors.

Conclusion The marked increase in AOP during the first week of storage suggests that RBCs are impacted early by the modification of their environment. The AOP behaviour reflects the changes in metabolism activity following the adjustment of the extracellular uric acid level. Knowing the origin, interdonor variability and the effects of the AOP on the RBCs could be beneficial for the storage quality, which will have to be further studied.

Key words: antioxidant, electrochemistry, erythrocyte, oxidative stress, storage, uric acid.

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Introduction

The storage of red blood cells (RBCs) under blood-banking conditions causes lesions that affect the quality of the product [1]. Recent clinical trials suggest that transfusing short-term stored erythrocyte concentrates (ECs) does not provide advantages compared to the transfusion of older ECs [2–4], although some reports describe deleterious effects in transfusion patients due to these age alterations [5–7]. Storage lesions [1, 8] may be either reversible and

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per se reversed by transfusion, such as the decrease in metabolism and protein activity [9–12], or irreversible, affecting protein integrity, phenotype and morphological properties of RBCs [13–15].

Oxidative stress is closely associated with these lesions [15–17] and the antioxidant (AO) defence system, which controls oxidation, plays an important preventive role. Likewise, an impaired AO defence induces a cascade of events related to protein function impairment, proteasome inhibition, oxidized protein accumulation at the RBC membrane and their elimination through microvesiculation [12, 18, 19]. The microvesicles released during storage [20, 21] could contribute to deleterious transfusion effects linked to haemostasis [22, 23]. The quality of RBCs following storage also depends on the donor's characteristics [24–26], including AO content, as a higher level of uric acid (UA, a major AO in plasma) contributes to the storability of RBCs [27].

Several approaches can be used for measuring the global AO activity in biological fluids, including spectrophotometric, fluorescence and electrochemical methods [28–31]. The electrochemical approach shows several advantages over the other methodologies. For instance, sample pretreatments are not required and measurements can be performed immediately after the electrochemical cell is loaded with the sample [32, 33]. Moreover, electrochemical measurements are not affected by background colours or autofluorescence, as occurs in most optical detection methods. In one electrochemical method, the current generated by the electrochemical oxidation of AOs dissolved in a liquid sample is measured. This process is based on the transfer of one or more electrons from the AO molecules to the biased working electrode (WE) of an electrochemical sensor (Fig. 1). The recorded current is proportional to the AO concentration. Each redox-active AO species is characterized by a different redox potential. Therefore, a linear potential ramp is applied to detect the full range of AOs in the sample. Such measurements might include the electrochemical detection of additional compounds in the sample which are also electrochemically active, but which do not show any relevant AO activity. Nevertheless, by their nature, most AO species, especially the ones of high biological importance, are electrochemically converted at low redox potentials with a high rate. Therefore, it is possible to distinguish the relevant AO signals from the signals not related to AOs using a mathematical treatment of the recorded curves which suppresses the measured signals at higher potentials. This procedure was introduced as electrochemical pseudo-titration voltammetry (vide infra) [32, 34].

Whereas the consequences of oxidative stress are well documented [1, 15–17, 35], the global AO content has been poorly quantified during RBC storage. We followed the global AO content of ECs and extracellular samples utilizing an electrochemical method. The measurement period started 1 day after blood donation and ended at day 43 (1 day after the expiration date for transfusion). Several trends which have not been reported elsewhere, such as an increase in the AO content in the EC and extracellular samples during the first days of storage, are presented and discussed.

Materials and methods

Preparation of the erythrocyte concentrates and samples

Six healthy donors (three women and three men) gave whole blood under signed consent at the blood centre of the Transfusion Interrégionale CRS, Epalinges, Switzerland [donor's characteristics are specified in supporting information (Table SI-1)]. ECs were prepared using a top/bottom processing method. Whole blood (450 ± 50 ml) was collected in 63 ml of citrate-phosphate-dextrose anticoagulant (Fresenius Kabi, CQ32250, Germany). After a holding time (between 2·5 and 4·25 h, depending of the donation) at a temperature of 22 ± 2°C, the bags were centrifuged (5047 **g** for 13 min at 22°C on a Roto Silenta 630 RS, Hettich, Switzerland) to separate blood components, and a semi-automated pressure was applied to distribute the fractions into sterile interconnected bags. Finally, the RBCs were filtered to remove residual leucocytes and were stored at 4°C in 100 ml of saline-adenine-glucose-mannitol (SAGM) additive solution at a haematocrit of 56·6 ± 1·6%.

EC samples (3 ml per bag) were collected daily from day 1 to 10, then weekly until day 43. Global AO power (AOP) and haematological data were measured on total ECs. Supernatants were separated from RBCs by centrifugation (2000 **g** for 10 min at 4°C) and used for extracellular AOP and haemolysis measurements. The remaining supernatants were saved at −80°C until quantification of the extracellular UA concentrations.

Haematological data and haemolysis measurements

Haematological data, such as the haematocrit (HCT) and the haemoglobin (Hb) concentration, were recorded with an automated haematology analyser (KX-21N, Sysmex, Switzerland). The concentration of Hb in the supernatant was determined according to the Harboe method with the 3-point 'Allen correction' by measuring the absorbance at 415, 380 and 450 nm with a spectrophotometer (NanoDrop 2000c, Thermo Scientific) [36, 37]. The percentage of haemolysis was then calculated using Eqn 1

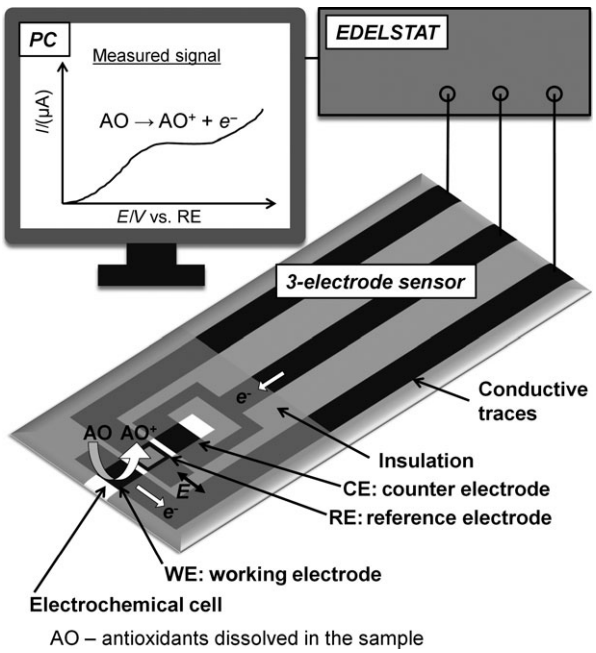

Fig. 1 Illustration of the electrochemical measurements of AOs using the 3-electrode disposable strips.

$$Haemolysis = \frac{Hb\ supernatant \times (1 - HCT)}{Hb\ total} \times 100 \quad (1)$$

where *Hb supernatant* is the concentration of Hb in the supernatant and *Hb total* is the concentration of Hb in the entire sample, both in g/l.

Colorimetric antioxidant power measurement

The extracellular AOP was quantified using a colorimetric method based on the decolourization of the green 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^{\cdot+}$), which converts into colourless ABTS by reduction with AOs. The bleaching rate is proportional to the AO concentration. Briefly, 80 μl of sample were mixed in a cuvette with 800 μl of acetate buffer (0·4 mM, pH 5·8) and 40 μl of $ABTS^{\cdot+}$ in acetate buffer (30 mM, pH 3·6), according to Erel's protocol [38], with slight modifications. The absorbance was measured (in duplicate) at 660 nm with a NanoDrop spectrophotometer. The assay was calibrated using ascorbic acid as a standard.

Uric acid quantification

One hundred and fifty μl of supernatants were transferred in Vivaspin 500 columns (Sartorius, Göttingen, Germany) and centrifuged at 15 000 g for 30 min at 4°C. Then, 30 μl of filtrates were dried (Genevac EZ-2 Mk3, Genevac Ltd, Ipswich, England) for 45 min and stored at −28°C. Just before analysis, the dried samples were dissolved in 90 μl of 45/55 (v/v) ammonium acetate (100 mM, pH8)/acetonitrile, vortexed, sonicated for 10 min at room temperature and stored on ice for 20 min. After a final centrifugation at 20 800 g for 30 min at 4°C, 45 μl of the supernatants were transferred into an injection vial.

One microlitre of sample was injected in duplicate on a high pressure liquid chromatography (HPLC) apparatus (Dionex UltiMate 3000 RSLCnano System, Thermo Scientific, Rockford, IL, USA). The capillary column (ZIC-HILIC, 150 × 0·3 mm, 5 μl, SeQuant Merck, Germany) was kept at 40°C. The chromatography conditions are summarized in Table SI-2 (solvent A was 100 mM ammonium acetate at pH 8 and solvent B 100% acetonitrile).

UA detection was performed by measuring its specific UV signal at 292 nm. Peak integration was calculated using the HyStar postprocessing software (Bruker Daltonics, Bremen, Germany). The nature of the detected compound was confirmed by mass spectrometry (Bruker Daltonics, amaZon ETD, negative ionization mode). Calibration was done for UA concentrations ranging between 0 and 75·0 μM (Fig. SI-1). The UA standard was dissolved in SAGM and prepared for injection in the same way as the samples.

Electrochemical antioxidant power measurement

Electrodes

Screen-printed electrode strips comprising a carbon paste WE, a Ag/AgCl quasi-reference electrode (QRE) and a carbon paste counter electrode (CE) were provided by EDEL-for-Life (Switzerland). The electrode areas (i.e. 1 mm × 1 mm for WE, CE and QRE) were defined precisely by a screen-printed dielectric insulation layer. On top of these electrode patterns, a spacer layer and a cover lid with two small openings on both sides of the sensor structure were created, forming a capillary. Hence, a thin, electrochemical cell is created, which is filled with the liquid sample by capillary forces within a fraction of second. This process leads to a reproducible electrode–sample interface.

Electrochemical apparatus, linear sweep voltammetry and electrochemical pseudo-titration voltammetry

Electrochemical measurements were performed at least in duplicate using an Edelmeter potentiostat (EDEL-for-Life, Switzerland). For each measurement, a fresh electrode strip was used and the electrochemical cell was filled with ~3 μl of total or extracellular EC sample. Three seconds after loading, linear sweep voltammograms (LSVs) were

recorded from 0 V to 1·2 V with a scan rate of 100 mV/s. All potentials herein are reported with respect to the integrated Ag/AgCl QRE. The electrode strip design allowed a rapid measurement, avoiding long exposure times of the samples to air and to the electrode surfaces.

Each LSV was transformed from a current value in ampere to an AOP in nW using the electrochemical pseudo-titration voltammetry [32, 34]. In brief, a Fermi-Dirac function was developed that contained curve points of unity at low potentials that became zero at higher potentials (Fig. SI-2). The measured current vs. potential curves, which start at zero current and that increase at higher potentials due to the presence of AOs, were multiplied by the Fermi-Dirac function resulting in a peak-curved shape. The AOP was then calculated by the integration of the obtained curves and represented a quantitative value to (1) compare the AOP of different samples and (2) to follow the AOP of each sample over time (Fig. SI-3).

Results

The six ECs exhibited standard haematological values over the storage period (Table SI-3), that is a slight decrease in RBC concentration and an increase in RBC size [14]. Haemolysis at the end of the storage period reached 0·18, 0·19, 0·20, 0·29, 0·25 and 0·21% for EC 1 to 6, respectively, which remained below the generally recommended value of 0·8%.

The calculated AOPs of the extracellular samples are plotted in Fig. 2a. All six samples exhibited a steep increase in the AOP during the first week of storage followed by a decrease until a plateau was reached. The extracellular AOP was linearly correlated with the AOP of the total EC (Fig. 3a). AOP levels in the six ECs were related to the sex of the donor. Indeed, the AOP was generally lower in women and remained lower during the storage compared to men (the integrated extracellular AOP was 5063 ± 349 day·nW for women and 6547 ± 668 day·nW for men, P-value = 0·027). Note that measurements in the bare storage solution (i.e. SAGM only) as a blank showed very low currents and demonstrated that all recorded signals in extracellular and EC samples were in majority due to the presence of soluble AOs coming from the residual plasma and RBCs (Fig. SI-4).

To quantitatively describe the observed trends, a kinetic model has been developed based on the following reactions:

$$AO_{pre} \xrightarrow{k_1} AO \xrightarrow{k_2} AO_{ox} \quad (2)$$

where AO_{pre} stands for precursors of AOs and AO_{ox} for the oxidized AOs. k_1 and k_2 are kinetic constants that represent the production and the consumption of AOs, respectively.

The AOP as a function of time is thus expressed considering 1st-order reactions as follows (SI-6 for details):

$$AOP(t) = AOP_0 e^{-k_2 t} + \frac{k_1}{k_2 - k_1} AOP_{pre,0}\left(e^{-k_1 t} - e^{-k_2 t}\right) + AOP_{pre,const}\frac{k_1}{k_2} \quad (3)$$

where AOP_0 and $AOP_{pre,0}$ are the AOPs at day 0 given in nW.

The measured AOPs were fitted to the equation derived from the kinetic model (Fig. 2b). The values obtained from the fitting procedure are shown in Table 1. Kinetic constant k_2 was lower than k_1, which is supported by the slow decay observed during storage. The efflux phase represented by k_1 was on average higher in women, and the second phase (slower and represented by k_2) was independent from the donor's sex.

The UA molecules significantly contribute to the AO defences of ECs. As a consequence, the UA concentration increased similarly to the AOP during the first week of storage (Fig. 4), reaching a maximum after 5 days for women and 7 days for men (maximum UA of 100·6 ± 22·6 μM for women and 147·1 ± 13·8 μM for men, P-value = 0·038). Men exhibited a higher UA concentration during the whole storage (area under the curve of 2687 ± 416 days·μM for women and 4231 ± 541 days·μM for men, P-value = 0·017). A plateau was reached after approximately 3 weeks for samples from women and 4 weeks for samples from men. The extracellular UA concentration and AOP were strongly positively correlated in a linear model (R^2 = 0·94, Fig. 3b).

The trends observed for the AOP measured with the electrochemical method and colorimetric assay (Fig. 5) were similar for both the early-storage trends and sex-related differences. However, a slight increase, instead of a plateau, was observed for the AOP in the measurements using the colorimetric assay at the end of storage. One hypothesis is that lipophilic AOs stemming from aged RBCs at the end of the storage were detected spectrophotometrically but not electrochemically due to their low electrochemical activity. It should be noted that a Hb concentration up to a level of 31 μM (i.e. 0·4% haemolysis) does not influence the electrochemical assay (Fig. SI-4) and that the colorimetric assay was corrected for Hb.

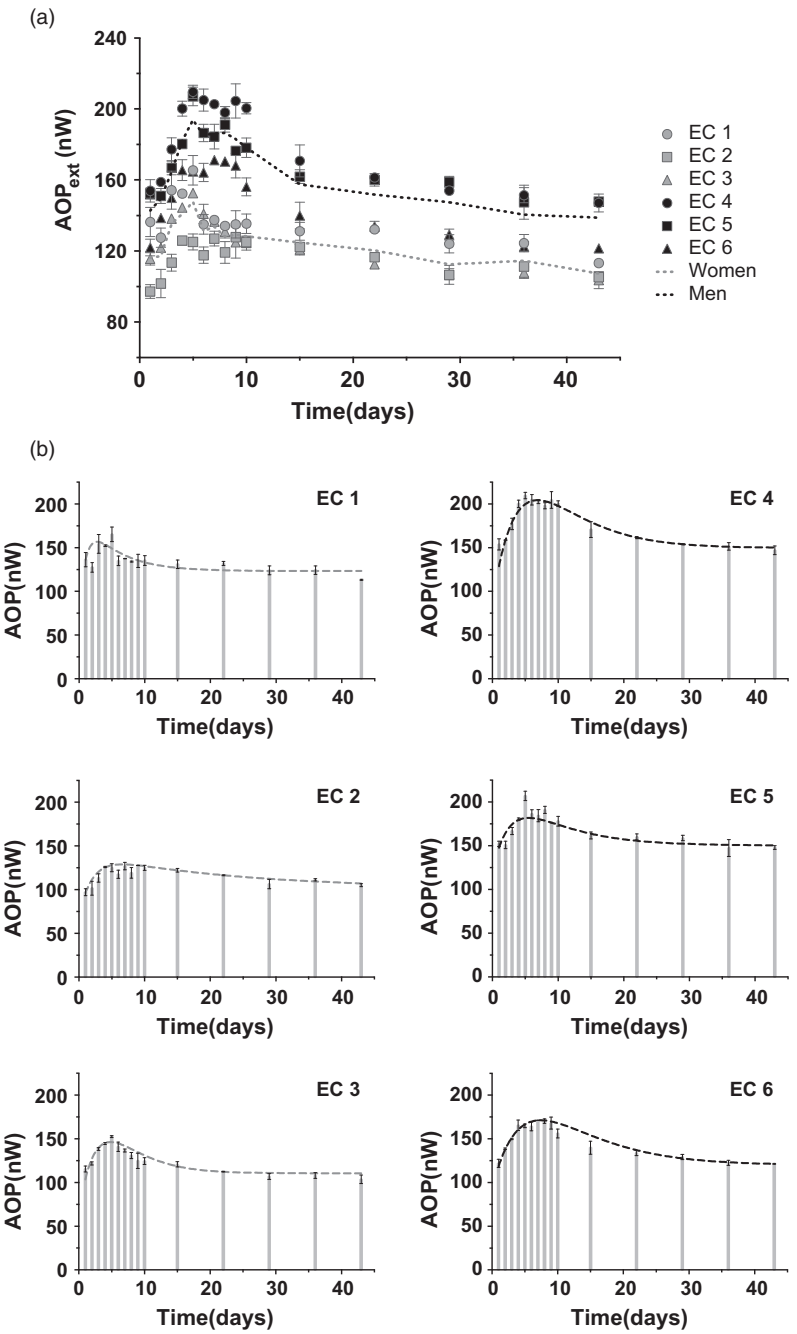

Fig. 2 Extracellular AOP (AOP$_{ext}$) measured with the Edelmeter for EC 1 to 6 over 43 days. (a) Extracellular AOP with individual and mean values (dotted line). (b) Extracellular AOP for individual ECs with curve fits for the kinetic model (dashed lines). Each measure was taken in duplicate or triplicate; error bars represent the SD.

Discussion

RBCs possess enzymatic (such as superoxide dismutase, catalase and peroxiredoxin) and non-enzymatic defences (UA, ascorbic acid, glutathione, etc.) that control reactive oxygen species, limit oxidative damage [18] and maintain a low level of metHb (a product of Hb autooxidation) [39]. The AOP measured in ECs was closely correlated to the extracellular concentration of UA, which is a known endogenous AO [40, 41], shown to account for 60-70% of the AOP in ECs [42].

The results obtained with the electrochemical and colorimetric AOP measurements suggest that the AO capacity of the investigated ECs is donor-dependent and impacted by changes in the RBC environment because of the blood processing. Of note, the type of additive solution used with variable composition, osmolarity and pH can impact differently the RBCs [43, 44]. Variations of the AOP

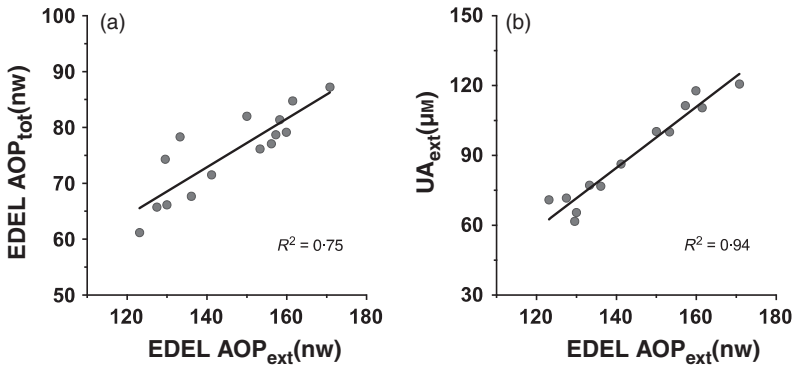

Fig. 3 (a) Correlation between total (AOP_{tot}) and extracellular AOP (AOP_{ext}) measured with the Edelmeter, and (b) between extracellular AOP and UA (UA_{ext}) concentration. Each point represents the mean value for six ECs at a different time. The linear correlation was calculated (R^2 regression coefficient).

Table 1 Fitting parameters for the extracellular AOPs

	Women			Men		
	EC 1	EC 2	EC 3	EC 4	EC 5	EC 6
AOP_0 (nW)	−49·153	−31·623	−47·338	−64·209	−29·940	−27·713
$AOP_{pre,0}$ (nW)	96·726	66·094	136·954	203·419	84·982	164·583
$AOP_{pre,const}$ (nW)	25·732	9·086	110·585	149·710	56·649	120·130
k_1 (per days)	0·955	0·495	0·279	0·191	0·356	0·157
k_2 (per days)	0·199	0·044	0·279	0·191	0·134	0·157

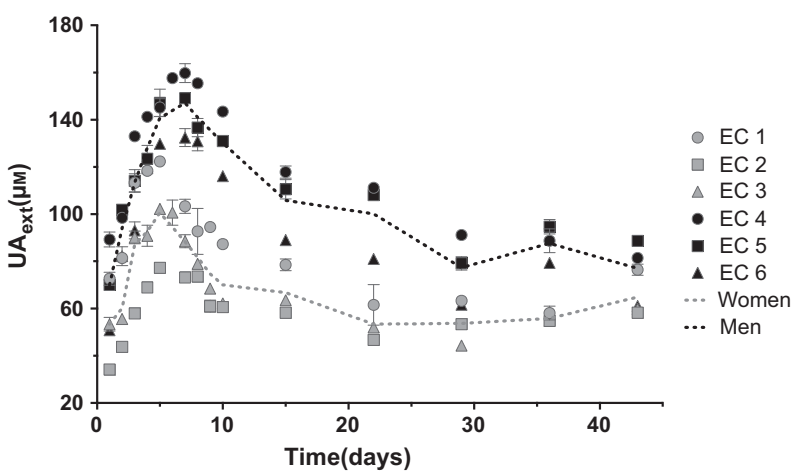

Fig. 4 Extracellular UA (UA_{ext}) concentration measured by HPLC. Samples were injected in duplicate; error bars represent ± the SD. The mean extracellular UA concentration was calculated for women and men (dotted lines).

values correspond to metabolic activity [10, 11] and are visible earlier compared to other ageing makers (i.e. haemolysis and microvesiculation, lactate accumulation, changes of morphology, etc.), which only become significantly visible after several weeks of storage [1, 8, 19].

The increase in AOP during the first week might result from a boost in RBC metabolism, as shown by the increased intracellular adenosine triphosphate (ATP) concentration during the first 10 days of RBC storage [9, 10]. Most probably, the evolution of AOP and UA concentration observed during the first week of storage is largely governed by an equilibration process between intracellular and extracellular media. Indeed, the residual plasma is diluted in the ECs because of the addition of SAGM to the RBCs. This is suggested by the steep increase in extracellular UA and the decrease in intracellular UA observed by Bordbar *et al.* [10]. In theory, the calculated dilution factor of plasma in the ECs is 11, even though an accurate measurement of the exact residual concentration needs to be standardized. Indeed, it is subject to strong variations (e.g. because of different blood processing methods), as pointed out by Jordan and Acker [45]. The

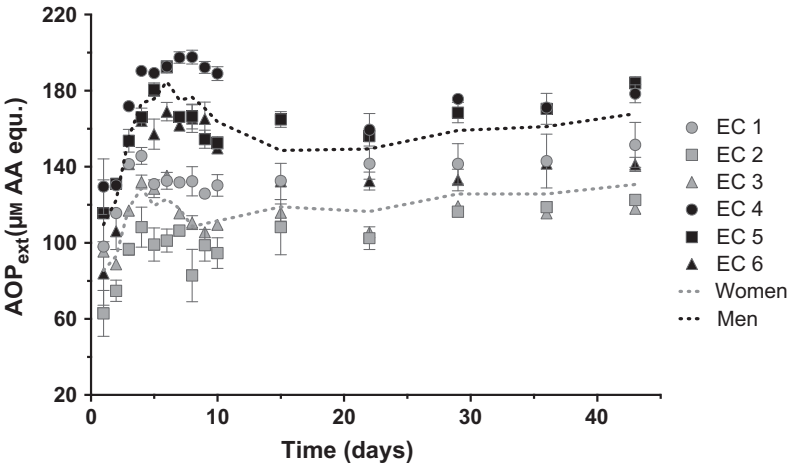

Fig. 5 Extracellular AOP (AOP_{ext}) measured with colorimetric assay, expressed as equivalent of ascorbic acid (AA). Each measure was taken in duplicate; error bars represent ± the SD. The mean extracellular AOP was calculated for women and men (dotted lines).

extracellular UA concentration, normally between 120 and 450 μM in human plasma, is subsequently diluted by the same factor. The dynamic of the UA concentration is governed by the active or passive transport of UA through the RBC membrane. Active UA transport is probably ATP-dependent [46] and membrane permeability for UA increases with storage time [47]. The balance between the intracellular and extracellular UA concentration was reached during the first week of storage. Interestingly, the increase in plasma AO activity was not observed during whole-blood storage (CPDA-1), where the analysis was based on a total radical-trapping AO parameter [48]. It corroborates our hypothesis that a change of storage environment induces changes in the RBCs.

In the second phase, the AOP decreased because the slowdown of glycolysis impairs the intracellular AO production (e.g. reduction in intracellular reduced glutathione after 2 weeks). Subsequent accumulation of oxidative species and/or re-entry of UA to protect the intracellular space against oxidative stress [27] could explain in part the decrease in extracellular UA concentration. Moreover, the purine pathway is no longer active as the adenosine monophosphate is transformed into inosine monophosphate, and consumed UA is not replaced [10, 11].

Finally, the time at which the AOP plateau is reached correlates with the reactivation of the purine metabolism (inhibition of the nucleotide salvage pathway). The feeding of the glycolysis, which is a result of the pentose phosphate pathway and the production of ribose-5 phosphate, produces hypoxanthine and xanthine. This process participates partially to the in situ synthesis of UA. This could explain why the extracellular UA concentration and AOP did not decrease further.

As demonstrated herein, the AOP level was related to the sex of the donor. Men had higher AOP than the women. This global AOP does not seem to be related to a lower haemolysis rate, although Jordan *et al.* reported that the haemolysis was lower in women [26]. The authors of that work also observed that buffy-coat processing induces less haemolysis than whole-blood filtering, suggesting that a higher amount of residual plasma results in less haemolysis. Therefore, we might speculate that female-derived ECs have more residual plasma and less haemolysis. This causality might be explained by the shift of UA following the dilution of plasma. It would be less important in female donors and impact the RBC metabolism less. In female donors, the capacity of RBCs to fight against storage lesions would consequently be better preserved. Nevertheless, this is beyond the aim of the present study and several other parameters can also explain the sex-dependent storability of RBCs, such as hormones [25, 49].

The kinetics of the AOP and the extracellular concentration of UA are in accordance with the three metabolic phases previously identified [10, 11]. Notably, the kinetic constants might demonstrate a donors' specific release of AOP (k_1) and a consumption of UA and other AOs (k_2), depending upon the storage conditions. This loss of AO capacity at the end of the storage corroborates the hypothesis and the cascade of events [7, 18, 19] that leads to the appearance of oxidative stress markers [13, 50] and oxidized molecules [15, 17, 18].

The kinetic model could be integrated into general kinetic analyses related to the RBC metabolism [51, 52] and therefore be used as predictive measure for the evolution of lesions. The presented approach could be a useful tool to understand RBC ageing and improve storage conditions. It has to be noticed that this value would probably not provide information on the quality of the RBCs and the level of lesions as the AOP was equivalent at the beginning and at the end of the storage. However, it could become a very useful indicator for how changes in the storage

solution, for example by adding AOs or by storing the sample under anaerobic conditions, can affect the AO defence of ECs, and how this can correlate to the ageing of RBCs to improve storage conditions.

Finally, the electrochemical method used enabled the rapid and reliable detection of hydrophilic AOs, in particular UA, in all six EC fractions. The similarity between total and extracellular AOP is due to the measured hydrophilic AOs that are present in the extracellular medium. The electrochemical measurements were performed with small sample volumes (<3 μl) within a few seconds and did not require any sample pretreatment, such as dilution. This technique has the capability to be a relevant tool for studying new storage solutions or to be considered as a quality control test, such as in platelet concentrates [33].

Batch fabrication techniques such as screen printing or inkjet printing allow the reproducible production of disposable electrode strips and enable electrode miniaturization [32, 53, 54]. Additionally, electrochemical workstations (i.e. potentiostats) are nowadays available in small and compact portable formats with wireless communication. As a result, electrochemical measurements can rapidly be performed at any location, for example in the field, in hospitals, at bedside or at home.

The implementation of sensors prepared by alternative advanced, large-scale production methods, such as inkjet printing, and the application of materials with higher activity towards the electrochemical detection of AOs, for instance using bare carbon nanotubes (CNTs) or CNTs covered with functional hydrogel layers [32, 55], could offer further possibilities for the rapid, low-cost and reliable monitoring of AOs in biological samples.

In conclusion, the AOP behaviour reflects the metabolic activity during the first 2 weeks of storage as previously reported [7, 10]. Our observations could be the result of the release of UA from the RBCs. The excretion of UA might disturb and displace the RBC metabolism through the purine salvage pathway during the first week of storage. Moreover, the sex has an impact and highlights the necessity to consider certain donor characteristics when evaluating the properties of the blood products and to potentially adapt the processing. The intracellular metabolism still needs to be studied in detail. Increasing the intracellular AOP defence and potentially postponing storage lesions could contribute to the improvement of the quality of blood products. The correlation between the AO levels in whole blood and in the derived ECs, and the quality of stored RBCs will be further studied.

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Author contributions

AL, FCS, JD, MP, PT and SM conducted the first experiments. MB and MP designed the experiments and wrote the manuscript. MB prepared and analysed the samples. AL and SM made the fittings and wrote the electrochemical part. JDT, HHG, NL reviewed the data and manuscript. All the authors read and approved the manuscript.

Conflict of interests

PT is an employee of EDEL-for-Life. The other authors declare no conflict of interests.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.
Appendix S1 Donor's characteristics.
Appendix S2 Uric acid quantification.
Appendix S3 Haematological data.
Appendix S4 Electrochemical pseudo-titration voltammetry.
Appendix S5 Linear sweep voltammograms revealing the AO content in the extracellular samples.
Appendix S6 Kinetic models for the antioxidant power development.