Supplementation of anti-oxidants in leucofiltered erythrocyte concentrates: assessment of morphological changes through scanning electron microscopy

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Introduction

Red blood cell (RBC) storage in the blood bank is associated with a sequence of biochemical, metabolic and mechanical changes leading to the progressive loss of cell viability and impairment of RBC survival upon transfusion into the recipient[1-6]. In detail, in erythrocytes stored for long periods, progressive haemolysis, alterations of permeability and flexibility, along with increased antigenicity are accompanied by an exacerbation of oxidative stress parameters, targeting both the lipid fraction (resulting in the accumulation of malonyldialdehyde and isoprostanes)[3] and the protein fraction (with increases in the levels of carbonylated species, protein fragmentation and non-enzymatically glycated haemoglobin)[3,5-11]. The storage-dependent accumulation of reactive oxygen species (ROS) is mainly related to the progressive loss of metabolic modulation[3,12], resulting in the impairment of anti-oxidant defences[6]. Indeed, in humans, each day about 3% of the body's haemoglobin undergoes spontaneous autoxidation to methaemoglobin because of the high concentrations of iron in RBC[6], whereby Fenton and Haber-Weiss reactions ultimately promote the accumulation of superoxyl and hydroxyl radicals, the latter being considered as the primary mechanism of injury in stored RBC[6].

It is worth noting that exacerbated vesiculation during the storage of packed red cells can also be regarded as a self-protective mechanism to eliminate irreversibly altered components, such as carbonylated proteins[13]. In this view, preventing the accumulation of oxidative stress has been long been regarded as a viable strategy to improve the quality of stored RBC. Over the years, several approaches have been proposed to achieve this aim: (i) supplementation of metal chelators, such as deferoxamine, diethylenetriaminepentaacetic acid or ethylenediaminetetraacetic acid, which bind iron released from ageing erythrocytes, thus protecting RBC from the Fenton and Haber-Weiss reactions[14]; (ii) oxygen depletion[15], to remove the main substrate of ROS-generating reactions and, in parallel, to improve the preservation of high energy phosphate compounds (adenosine triphosphate and diphosphoglycerate), at the expense of anti-oxidant defences[16], by relying on the oxygen-dependent modulation of metabolic fluxes according to the model proposed by the groups of Low and Giardina[17,18]; (iii) supplementation of anti-oxidants in the additive solutions, using compounds containing thiol groups (glutathione loading)[19] and vitamins (especially C in the form of ascorbate/dehydroascorbate and vitamin E)[20-24].

This last strategy has attracted a great deal of interest during recent decades, especially in the light of the central role of glutathione (GSH) in intracellular anti-oxidant systems. Indeed, glutathione is the substrate for three key RBC anti-oxidant enzymes: glutathione peroxidase, glutathione reductase and glutathione S-transferase. Glutathione peroxidase-catalysed reactions inactivate H_2O_2 to neutral substances, water and oxidized glutathione (GSSG). Therefore, ROS are not generated in glutathione reactions, as is the case for superoxide dismutase (SOD)-catalysed reactions, from which it can be inferred that glutathione-related enzymatic mechanisms, rather than other enzymatic mechanisms, are primarily committed to protection against the auto-oxidation of erythrocytes[25].

Oxidized glutathione is constantly being reduced by glutathione reductase through its utilization of NADPH, which is mainly generated via the pentose phosphate pathway. However, it has been recently confirmed that the progressive loss of metabolic modulation over storage proportionally impairs the capacity of RBC to replenish NADPH reservoirs via the pentose phosphate pathway, especially after the second week of storage[2,12]. In the light of this, alternative strategies could be pursued:

- to preserve GSH levels by promoting *de novo* synthesis of this tripeptide by fuelling a key limiting substrate precursor, cysteine, in the form of N-acetylcysteine (NAC)[26];

or

- to reduce GSSG back to GSH, via reactions involving dehydroascorbate/ascorbate (vitamin C)[27,28].

Recently, we performed an extensive mass spectrometry-based metabolomics analysis to understand whether vitamin C and NAC supplementation improved

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storage quality, especially in terms of anti-oxidant potential and glutathione homeostasis and energy metabolism (Pallotta et al., *unpublished data*). Encouraging results suggested that loading of anti-oxidants boosted GSH levels and helped to prevent malondialdehyde accumulation and reduce haemolysis. However, we could not conclude whether anti-oxidants also preserved RBC morphology better during storage. In the light of the foregoing considerations, we investigated whether supplementation of ascorbate and NAC in the SAGM additive solution helped to preserve RBC morphology better throughout a 42-day storage period.

Materials and methods

Sample collection

Red blood cell units were drawn from healthy donor volunteers in accordance with the policy of the Italian National Blood Centre guidelines (Blood Transfusion Service for donated blood) and after informed consent in conformity with the declaration of Helsinki. We studied RBC units collected from 10 healthy male donor volunteers (age 39.4±7.5 [mean±SD] years). Leucofiltered (log_4) packed red cell units were stored under standard conditions at 4 °C for up to 42 days (i) in the presence of CDP-SAGM, or (ii) in CPD-SAGM with the addition of ascorbic acid (0.23 mM-Sigma Aldrich, Milan, Italy) and NAC (0.5 mM-Sigma Aldrich). Dosing experiments for ascorbic acid and NAC were performed to minimise haemolysis at the end of the storage period. Sterility was assessed throughout the whole storage period.

Samples were removed aseptically for the analyses on a weekly basis. Samples for metabolomics studies were collected at 0, 7, 21, 28 and 42 days of storage.

Scanning electron microscopy

Scanning electron microscopy (SEM) studies of the control and treated RBC were performed using a JEOL JSM 5200 electron microscope on days 0, 28 and 42. The samples were prepared and the images analysed as previously reported[4]. SEM images were assembled with Photoshop CS 5.1 (Adobe Systems, Mountain View, CA, USA).

Results and discussion

Electron microscopy analyses evidenced substantial improvements in red blood cell morphology only in the first 28 days of storage

Storage in the presence of vitamin C and NAC improved the morphology score within the first 4 weeks of storage (Table I). However, supplementation with vitamin C and NAC did not produce any significant improvements at the end of the storage period (Table I). Indeed, at 42 days, the percentage of discocytes in supplemented units was not significantly higher than that in untreated controls (24.2%±2.1% *vs* 21.8%±1.6%; Table I), although the percentage of irreversibly altered RBC (including sphero-echinocytes, spherostomatocytes, spherocytes and degenerated shapes) was lower than in controls (29.5%±3.6% *vs* 34.6%±3.2%).

Table I - SEM erythrocyte shape classification

Storage day	Discocyte (%)	Reversibly* changed RBC (%) (echinocyte and stomatocyte shape)	Irreversibly* changed RBC (%) (sphero-echinocyte, spherostomatocyte, spherocyte, valocyte, and degenerated shapes)
0	77.3±2.9	18.4±4.6	4.3±3.1
28 Control	46.1±3.8	36.2±1.4	17.7±2.6
28 Vitamin C+NAC	51.8±1.3**	32.6±2.9**	15.5±1.8
42 Control	21.8±1.6	43.6±2.7	34.6±3.2
42 Vitamin C+NAC	24.2±2.1	46.3±1.9	29.5±3.6

* Reversible and irreversible changes were classified based on previously reported criteria[6,9]

**Statistically significant (p-value<0.05 ANOVA in comparison to untreated controls at the same storage time point).

Figure 1 shows a mosaic of SEM images, including control RBC stored in CPD-SAGM at days 0, 28 and 42 (Figure 1A, B and C, respectively), and RBC supplemented with vitamin C and NAC at the same times (Figure 1D, E and F, respectively). While the majority of red blood cells in supplemented units were still discocytes at 28 days (51.8%±1.3%; Table I, Figure 1.G), unaltered discocytes represented a minority of the RBC population and perfectly disc-shaped phenotypes were almost totally absent at the end of the storage period (Figure 1H).

A direct comparison of these results against those of our recent study on changes in RBC morphology arising upon deoxygenation[29] suggests that oxygen removal might preserve RBC morphology better than the hereby-discussed supplementation of anti-oxidants.

It is worth recalling that an increase in intracellular calcium directly induces or indirectly promotes a cascade of molecular and cellular events such as loss of deformability, and echinocytosis[30], besides protein degradation/cross-linking, recognition signalling and, ulitmately, eryptosis[31]. This is particularly true in units that have not been leucofiltered[31], which exacerbates oxidative stress. Indeed, oxidative stress is increasingly

emerging as an additional trigger to calcium-dependent morphological lesions of RBC[32], which likely results from the close cross-talk between energy and redox metabolism in RBC, especially in the blood bank[2]. Future investigations might, therefore, be designed to test whether metabolic (Pallotta *et al.,* unpublished data) and morphological (present study) changes observed in RBC stored with anti-oxidant supplementats (vitamin C and NAC) might be correlated with alterations in intracellular calcium reservoirs.

Conclusion

Although oxidative stress represents a critical challenge for RBC under blood bank storage conditions, boosting redox metabolism alone does not seem to be sufficient to completely prevent and cope with the lesions targeting membrane morphology. On the other hand, when compared to untreated controls, supplementation of anti-oxidants helped to preserve RBC morphology for a broader shelf-life window (the first 4 weeks of storage), which suggests that supplementation of anti-oxidants might be a viable strategy to improve storage quality, rather than to extend storage duration of erythrocyte concentrates.

Future investigations might address the effects of the combination of deoxygenation and anti-oxidant supplementation strategies on storage quality of erythrocyte concentrates.

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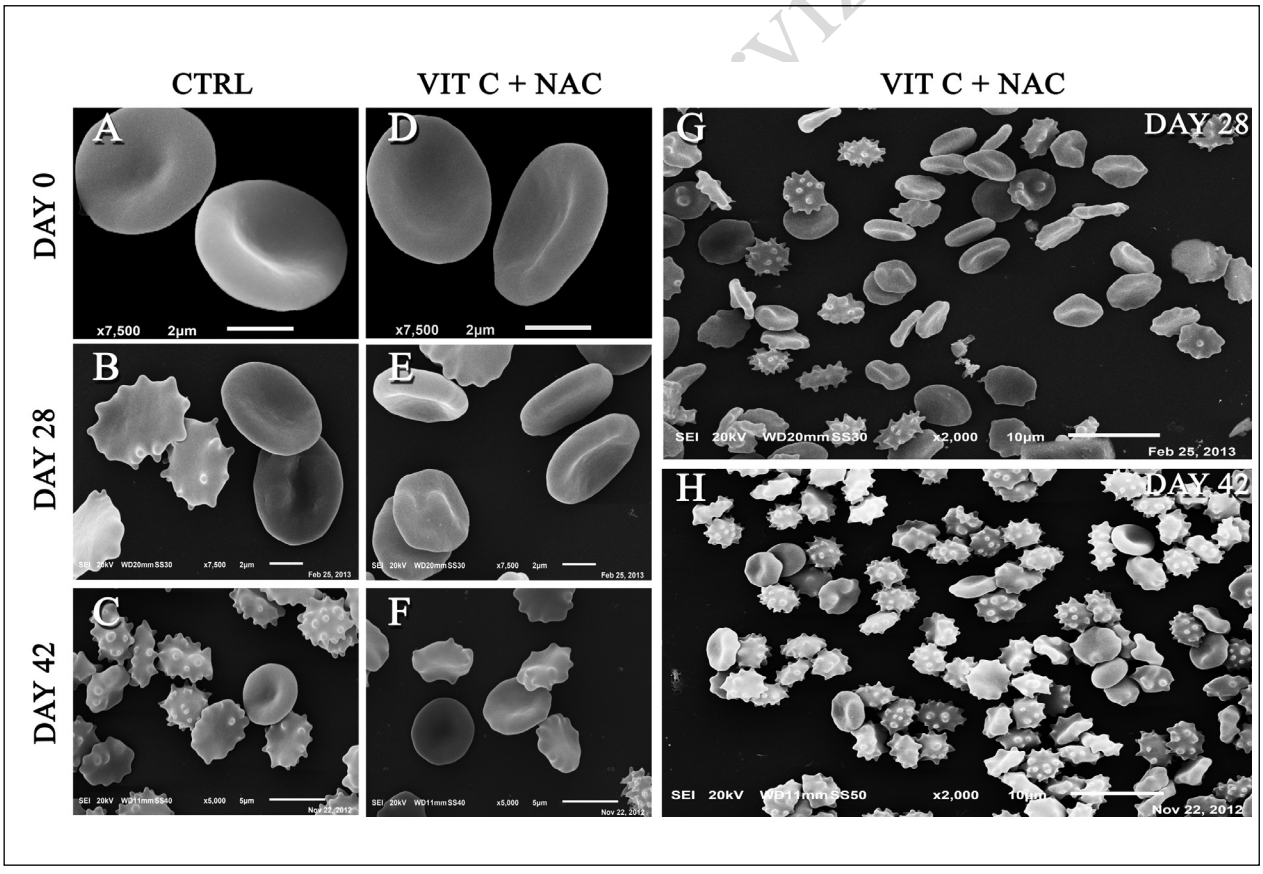

Figure 1 - Scanning electron micrograph of control red blood cells (day 0 - A; day 28 - B; day 42 - C), and vitamin C+NAC-supplemented red blood cells (day 0 - D; day 28 - E; day 42 - F). In E and F, 2,000x magnification of vitamin C+NAC-supplemented red blood cells at 28 and 42 days, respectively. Scale bars and magnification values are reported in each panel. Panels were assembled with Photoshop CS 5.1 (Adobe Systems, Mountain View, CA, USA).

The Authors declare no conflicts of interest.

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