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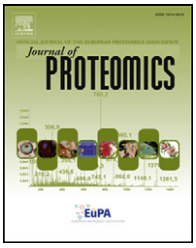

Effects of pre-storage leukoreduction on stored red blood cells signaling: A time-course evaluation from shape to proteome☆

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ABSTRACT

The introduction of pre-storage leukoreduction in the preparation of standard RBCs intended for transfusion provided significant improvement in the quality of labile products and their post transfusion viability and effects, although the literature data are controversial. To elucidate the issue of the probable leukoreduction effects on RBCs storage lesion, we evaluated various storage quality measures in RBCs stored in either leukoreduced (L) or non-leukoreduced (N) units, with emphasis to senescence and oxidative stress associated modifications. Our data suggest that the residual leukocytes/platelets of the labile products represent a stressful storage factor, countering the structural and functional integrity of stored RBCs. Hemolysis, irreversible echinocytosis, microvesiculation, removal signaling, ROS/calcium accumulation, band 3-related senescence modifications, membrane proteome stress biomarkers as well as emergence of a senescence phenotype in young RBCs that is disproportionate to their age, are all encountered more or mostly in N-RBCs compared to the L-RBCs, either for a part or for the whole of the storage period. The partial, yet significant, alleviation of so many storage-related manifestations in the L-RBCs compared to the N-RBCs, is presented for the first time and provides a rational mechanistic interpretation of the improved storage quality and transfusions observed by the introduction of pre-storage leukoreduction.

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Abbreviations: BS3, bis-sulfosuccinimidyl-suberate; CSLM, confocal laser scanning microscopy; CPD, citrate-phosphate-dextrose; CMH2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichloro-dihydro-fluorescein diacetate, acetyl ester; DCF, dichlorofluorescein; Fluo-4AM, fluorescent calcium indicator Fluo-4; Hb, hemoglobin; HCT, hematocrit; L, leukoreduced; MCH, mean cell Hb; MCHC, mean cell Hb concentration; MCV, mean cell volume; N, non-leukoreduced; NEM, N-ethylmaleimide; NS, non-stored; PCI, proteome carbonylation index; PS, phosphatidylserine; Prx2, peroxiredoxin 2; RBCs, red blood cells; RDW, red cell distribution width; SAGM, saline-adenine-glucose-mannitol; S-RBCs, high-density fractions of RBCs enriched in senescent-RBCs; t-BHP, tert-butyl hydroperoxide; Y-RBCs, low-density fractions of RBCs enriched in young RBCs.

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1. Introduction

Effective *ex vivo* storage of red blood cells (RBCs) is an essential requirement for medical practice. Stored RBCs undergo a series of time-dependent – yet early enough recognizable – physiological, structural and biochemical alterations, which are only reversible to some extent [1]. In the RBC storage lesion context, physiologically important disturbances in energy metabolism, rheological properties (shape, deformability, aggregability, intracellular viscosity), oxidation/carbonylation stress and finally, in cellular aging process have been widely characterized [1,2]. Altered membrane surface and cytoskeleton contribute to the RBCs damage and clearance [3,4]. Although the clinical importance of the RBCs storage lesion is poorly understood, some of the irreversible deteriorations of the stored RBCs, like hemolysis, potassium release and microvesicles accumulation, are associated with reduced post-transfusion survival/efficacy and increased risk of adverse reactions in the recipients [5,6].

The currently used techniques for the preparation of standard RBCs products include the pre-storage filtration of blood to remove the contaminating donor leukocytes and platelets. Despite controversy among the randomized clinical trials on the beneficial or neutral effects of filtration [7,8], the introduction of pre-storage leukoreduction has provided significant improvement in transfusions by lowering the incidence of viruses transmission and the circumstantial, yet severe, untoward clinical effects (alloimmunization, immunosuppression, inflammatory responses etc.) that have been related to increased morbidity/mortality liability [5,9]. Moreover, the leukoreduction exhibits a beneficial effect on RBCs storage lesion, by improving both the hemolysis and the post-transfusion recovery of leukoreduced RBCs [9,10]. Activated, apoptotic or degenerated leukocytes could equally trigger adverse transfusion reactions and storage-associated damage, since they represent a source for bioactive factors, like oxygen free radicals, cytokines and enzymes [10,11]. However, pre-storage leukocyte reduction has not eliminated all the leukocyte-related responses [12] nor the biochemical and morphological changes that occur to RBCs as a consequence of aging and storage [13]. As a matter of fact, some authors claimed that there is but a modest improvement in post-infusion viability of leukoreduced RBCs vs. the non-leukoreduced ones [14]. Further on, a recent study suggested that the pre-storage filtration may aggravate blood storage lesions [15]. Finally, although leukoreduction has been assigned as a standard in the labile products making, today, approximately 20% of the transfusions in the United States [16] and much higher percentage of those performed in several European countries are still conservative, namely non-leukoreduced. Therefore, there is still a need for clarifying the probable effects of leukoreduction on storage lesion progression as well as on *in vivo* efficacy and adverse transfusion effects. Adequate inventory and understanding of RBCs storage lesion in relation to various storage strategies currently followed in clinical practice is probably the only way to mitigate the disturbances that render RBC transfusions dangerous or less effective.

This study aims at reporting experimental facts touching the effect of residual leukocytes in the progression of RBCs storage lesion under standard blood banking conditions, with emphasis to the senescence and oxidative stress associated molecular and cellular modifications. To this purpose, we evaluated a series of storage quality measures in RBCs stored in either leukoreduced (L) or non-leukoreduced (N) units. A careful standardization of sample harvesting, process and storage was set up in order to minimize the pre-analytical variations. Our hematological, structural, biochemical and proteomic data suggest that the residual leukocytes and platelets represent an additional stressful factor for the stored RBCs, affecting almost all RBCs removal signaling mechanisms and thus their structural and functional integrity. The partial, yet significant, alleviation of so many storage-related manifestations in L-RBCs compared to N-RBCs is presented for the first time and provides a rational mechanistic interpretation of the improved storage quality and transfusions observed after the introduction of pre-storage leukoreduction.

2. Materials and methods

2.1. *Material supplies*

Antibodies against band 3, spectrin, human IgGs and HRP-conjugated antibodies to goat IgGs, as well as protease inhibitor cocktails, phosphatase inhibitor cocktail 2, t-butyl hydroperoxide (t-BHP) alpha-cellulose, microcrystalline cellulose (type 50), N-ethylmaleimide (NEM), Percoll medium (d = 1.13 g/ml), Drabkin's reagent components and common chemicals and buffers were all obtained from Sigma–Aldrich (Munich, Germany). Electron microscopy grade glutaraldehyde solution was from Serva (Heidelberg, Germany). Antibodies against hemoglobin (Hb) and peroxiredoxin 2 (Prx2) were obtained from Europa Bioproducts (UK) and from Acris GmbH (Herford, Germany), respectively. Antibodies against CD47, clusterin, HSP70, calpain-1 (μ-calpain), cathepsin E and band 3 were from Santa Cruz Biotechnology (CA, USA). Anti-human CD59 and HRP-conjugated anti-rabbit IgG were from R&D Systems (MN, USA). mAbs against synexin (annexin VII) and flotillin-2 were obtained from BD Transduction Laboratories (CA, USA). Sodium orthovanadate and Syk inhibitors II and IV were from Calbiochem (Darmstadt, Germany). bis-sulfosuccinimidyl-suberate (BS3) crosslinker was from Thermo Scientific (Rockford, IL). Anti-phosphotyrosine (pTyr, clone PY20) mAb, MF membrane syringe driven filters and the Oxyblot® detection kit were obtained from Millipore (Temecula, CA). A-23187 ionophore and annexin-V-Fluos solution were from Roche Diagnostics (Burgdorf, Switzerland). 5-(and-6)-chloromethyl-2′,7′-dichloro-dihydro-fluorescein diacetate, acetyl ester (CMH2DCFDA) and fluorescent calcium indicator Fluo-4 (Fluo-4 AM) were from Invitrogen, Molecular Probes (Eugene, OR). HRP-conjugated antibodies to rabbit IgGs, ECL Western blot detection kit and Percoll solution (d = 1.131 g/ml) were from GE Healthcare (Buckinghamshire, UK). HRP-conjugated antibodies to mouse IgGs were from DakoCytomation (Glostrup, Denmark). Rabbit anti-human CD235a was from AbD Serotec (Oxford, UK). Bradford protein assay was obtained from Bio-Rad (Hercules, CA). Western lighting Plus ECL was from Perkin Elmer (CA, USA). mAb against stomatin and antiserums against proteins 4.1R and pallidin (band 4.2) were kindly provided by Prof. R. Prohaska (Institute of Medical