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COMPARATIVE EVALUATION OF STORAGE LESIONS INDUCED BY ANTICOAGULANT MEDIUM ON BLOOD FOR TRANSFUSION

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ABSTRACT: Numerous cellular changes occur during RBCs storage in blood banks. Morphological and physiological changes resulting from the storage of RBCs are known as "storage lesions". These alterate RBCs normal biology and functionality, affects both oxygen transport efficiency and other additional risks of disruption of the immune system and coagulation. The similarity between red blood cells senescence phenotype and one of cells in apoptosis, rise to the hypothesis that the accelerated RBCs senescence may be an apoptosis phenomenon even for the particular case of human erythrocytes, cells lacking in mitochondria and nucleus.

Thus, we evaluated the storage lesions induced by anticoagulant medium of RBCs for transfusion. The results of our researches revealed the involvement of an apoptosis phenomenon in accelerated aging of red blood cells in blood bags as the basic phenomenon of storage lesions. Also, our results have validated our new proposed cell viability and apoptosis detection assays by flow cytometry to explore the quality of stored blood components for transfusion after blood storage.

Keywords: RBCs, transfusion, storage lesions, apoptosis, ROS, viability, flow cytometry

INTRODUCTION:

RBCs transfusion is the most common therapeutic procedure performed to improve oxygen transport to tissues. Based on current regulations, RBCs units for transfusion may be stored to 42 days at refrigerated temperature before transfusion. Despite a century of improvements in the field of RBCs storage, biomedical evidences, including proteomic and metabolomic investigations suggests that storage duration might be related to a progressive accumulation of changes, so-called "storage lesions" (Tinmouth *et al*., 2006; Kriebardis *et al.,* 2008; Hess, 2010; Cohen & Matot, 2013; D'Alessandro & Zolla, 2013). Alterations of morphology, membrane proteins or lipid composition of the membrane, with the preferential loss of certain classes of lipids and enrichment of others, such as glycerophosphoserines and ceramides (Timperio *et al.,* 2013; Almizraq *et al.,* 2013), compromise the survival of transfused, long-stored erythrocytes, since as many as 25% of the transfused RBCs do not survive more than 24 hours upon transfusion into the bloodstream of a recipient (Veale *et al.,* 2014; Bosman 2013).

Until today, the nature of storage lesions has not been fully understood (Beutler *et al*., 2000). A major hypothesis suggests an accelerated phenomenon of RBCs senescence, resulting in recognition of them as "non-self" and rapid elimination from circulation (Messana *et al*., 2000; Bratosin *et al.,* 2002). Despite extensive physiological data, indications to plead the need for transfusion are still controversial. Transfusion of RBCs are administrated most often in patients who have undergone surgery or are in ICU (Vincent *et al*., 2002; Rao *et al*., 2002; English *et al*., 2002; Shapiro *et al*., 2003). Those observations focused on the idea that stored RBCs may not deliver enough oxygen to critically ill patients. Some studies suggest that storage lesions may be responsible for complications associated with transfusion, as immunosuppression and "multiple organ failure" syndrome (Purdy *et al*., 1997; Offner *et al*., 2002) and other clinical consequences of this phenomenon.

Although identified for a long time, apoptosis or programmed cell death is now regarded as the primary physiological mechanism that regulates the life of the cell and serves to control the elimination of unwanted cells. Apoptosis was long time attributed exclusively to nucleated cells. This hypothesis was so strong that took more than 20 years to recognize apoptosis in an anucleated cell's cytoplasm (Schulze-Osthoff *et al*., 1994; Jacobson *et al*., 1994; Martin *et al*., 1996; Castedo *et al*., 1996).

Daily, about 360 milliards human red blood cells are eliminated from circulation (3-4 million/second) after 120 days, in the absence of any inflammatory phenomena, providing the body homeostasis. The similarity between red blood cells senescence phenotype and one of cells in apoptosis, rise to the hypothesis that the RBCs senescence may be an apoptosis phenomenon even for the particular case of human erythrocytes, cells lacking mitochondria and nucleus. In 1999, for first time, Bratosin and his collaborators reported that the programmed cell death (PCD) of human erythrocytes induced by Ca^{2+} influx is prevented by protease inhibitors and hypothesized for first time that phagocytosis of senescent RBCs occurred at the end of an apoptotic phenomenon (Bratosin *et al.,* 1999). This view was confirmed two years later by Bratosin (Bratosin *et al.,* 2001) and Berg (Berg *et al.,* 2001) who reported simultaneously that the programmed cell death of RBCs could be induced by Ca^{2+} influx and prevented by caspase and calpain inhibitors. A few years later, the same group clearly

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demonstrated the presence of active caspases 8 and -3 among the fraction of circulating erythrocytes displaying phosphatidylserine residues on their surface isolated from circulating RBCs (Bratosin *et al.,* 2009). Consequently, they indicate that mature erythrocytes share with all other mammalian cell types the capacity to self-destruct in response to environmental signals .

In the present study, to evaluate the storage lesions induced by acidic anticoagulant medium on preserved blood for transfusion, we analyzed the apoptosis phenomenon of erythrocytes using new proposed methods and criteria by flow cytometry (Bratosin *et al.,* 1998; Bratosin *et al.,* 2001; Bratosin *et al.,* 2005; Mitrofan-Oprea *et al.,* 2007)

MATERIALS AND METHODS:

Chemicals

The fluorogenic dye Calcein acetoxymethyl ester (Calcein-AM) and 2´,7´-dichlorofluorescein-diacetat (DCFH-DA) were from Sigma Aldrich (Saint Louis, Mo, USA) and fluorescein-conjugated Annexin-V was purchased from Pharmingen (San Diego, CA,USA)

Erythrocytes collection and preparation of RBCs storage systems

Human blood type 0 Rh+ used in all experiments was supplied by Bucharest Hematology Center and Army Transfusion Center, and was conducted in accordance with protocols of blood preservation from blood banks. 450 mL of blood were taken by the conventional puncture in 63 mL of CPD anticoagulant solution (Citrate-Phosphate-Dextrose), pH 5.5 with the following composition: citric acid 3.25 g, sodium citrate 26.30 g, glucose 25.50 g, monosodium phosphate 2.51 g and 1000 mL pyrogen free water (q.s.p.). Blood was also collected on heparin.

The concentrated of red blood cells (CGR) was obtained after 24 hours of storage at 20 °C by deleucocytation with Leucoflex LST (MacoPharma Patent) filter and brought to a 60% hematocrit with a SAGM (Sodium Adenine Glucose Mannitol) nutrient additive solution pH 5.1 ± 0.3 (sodium chloride 8.770 g, adenine 0.169 g, glucose (monohydrated) 9.000 g, manitol 5.250 g and 1000 mL pyrogen free water (q.s.p.). Storage bags were performed at 4°C for 42 days (6 weeks).

Flow cytometric analysis

Flow cytometric analyses were performed on FACScan cytometer (Becton Dickinson, San Jose, CA, USA) using CellQuest Pro software for acquisition and analysis. Cells in suspension in isotonic PBS buffer pH 7.4 were gated for the light scatter channels on linear gains and the fluorescence channels were set on a logarithmic scale, a minimum of 10000 cells being analyzed in each condition. The results were expressed as MFI value of the logarithmic fluorescence intensity.

Determination of phosphatidylserine exposure using Annexin-FITC

Erythrocytes were resuspended (10^6 cells) in HEPES binding buffer pH 7.4 containing 2.5 mM calcium chloride with 10 µL (0.1µg) of Annexin-V-FITC solution and incubated for 15 min at room temperature in the dark. The cells were gated for biparametric histograms FL1 (FITC fluorescence) versus FL2 (RBC autofluorescence) using quadrant technique. Experiments were carried out in triplicate.

Reactive Oxygen Species measurement

Intracellular level of reactive oxygen species was measured using an oxidation-sensitive fluorescent probe, 2´,7´-dichlorofluorescein-diacetat (DCFH-DA) according to method of Bass *et al.,* 1983.

In the presence of various intracellular reactive oxygen species, 2´,7´-dichlorofluorescein-diacetat (DCFH-DA) is oxidized to the highly fluorescent compound, 2´,7´-dichlorofluorescein (DCF) respectively. A working solution with a concentration of 500 µM was prepared extemporaneously from 100 mM H_2DCFH-DA stock solution in DMSO. The erythrocytes ($5x10^5$/mL PBS) were incubated for 1h at 37 °C with 5 µM DCFH-DA dissolved in DMSO. The appearance of reactive oxygen species was measured by flow cytometry for the green fluorescence (FL1) using an excitation and emission settings of 488 and 530 nm respectively compared to a positive control that had been incubated with 2mM H_2O_2 to stimulate ROS production in RBCs and consequently with a cellular positive fluorescence.

RBCs viability assessment using Calcein-AM

Cell viability assessment was studied according to the procedure of Bratosin *et al.* 2005 based on the use of acetoxymethyl ester calcein (Calcein-AM), a fluorescein derivative and non-fluorescent vital dye that is converted by cytosolic esterases into green fluorescent calcein which is retained by cells with intact membranes. The membrane-permeable dye Calcein-AM was prepared as a stock solution of 10 mM in dimethylsulfoxide stored at -20 °C and as a working solution of 100 µM in PBS buffer pH 7.4. RBCs ($4 x10^5$ in 200 µL PBS buffer, pH 7.4) were incubated with 10 µL Calcein-AM working solution

(final concentration in Calcein-AM: 5 µM) for 45 min at 37 °C in the dark and then diluted in 0.5 mL of PBS buffer for immediate flow cytometric analysis of calcein fluorescence retention in cells. Experiments were performed at least three times with three replicates each time.

RESULTS AND DISCUSSION:

Flow cytometric assessment of the extracellular pH influence from anticoagulant medium on phosphatidylserine externalization determinated with Annexin-V-FITC

A first change that occurs in cellular apoptosis is changing the membrane symmetry with translocation of phosphatidylserine (PS) from the cytoplasmic face to the exterior of the membrane (Diaz & Schroit, 1996). This assessment can be made by the use of Annexin-V, a 35-36 kD protein, which is capable to recognize with a high affinity the PS residues and bind them in the presence of calcium ions. Annexin-V marking with fluorochrome such as FITC, allows its use as a highly sensitive tool to identify cells in

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apoptosis by flow cytometry.

As shown in figure 1, the identification of viable and apoptosis cells, was analyzed by the quadrants technique with Annexin-V-FITC (FL1) versus autofluorescence (FL2) during preservation for 4 weeks in SAGM medium for highlighting the influence of the acidic pH (pH 5.6) of the CPD sampling medium compared with heparin (Fig. 2).

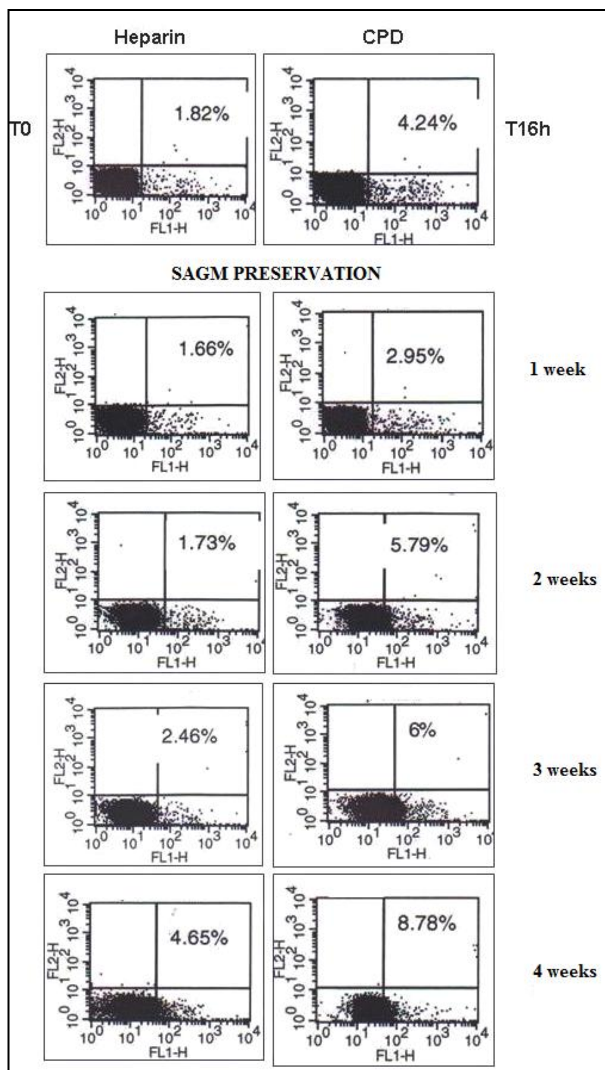

Fig.1 Flow cytometric analysis of the acidic pH (pH 5.6) influence of the CPD anticoagulant medium during storage for four weeks in SAGM medium. Identification of viable and apoptotic cells was made by quadrants technique with Annexin-V-FITC (FL1) versus autofluorescence (FL2). Abscissa: green fluorescence intensity of Annexin-V-FITC (FL1), in logarithmic scale. Ordinate: autofluorescence intensity in logarithmic (FL2). Bottom left quadrant: viable red blood cells (Annexin$^-$); Lower right quadrant: red blood cells under apoptosis (Annexin$^+$); The percentage of cells refers to the percentage of apoptotic cells (Annexin$^+$). Number of analyzed RBCs: 10000. Results are representative of three experiments performed.

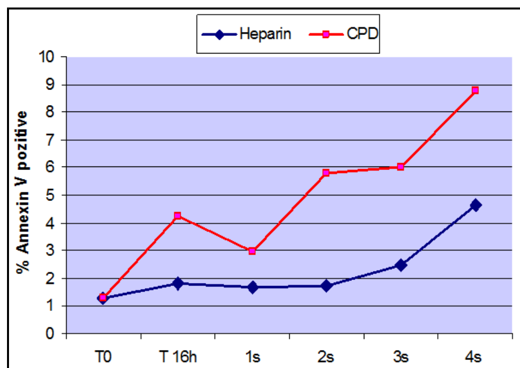

Fig. 2 Comparative histogram of externalization degree of phosphatidylserine exercised from the acidic pH (pH 5.6) of the CPD anticoagulant medium compared with heparin during storage for four weeks in SAGM medium, based on the data from quadran technique analysis with Annexin-V- FITC (FL1) versus autofluorescence (FL2) shown in figure 1. The results shown are representative of two experiments performed.

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It is easier to see that after 16h, 4.24 % of the blood collected on acidic CPD medium (5.6 acidic pH) has externalised PS compared to just 1.82 % on blood collected onto heparin

Further, during the same conservation on the same SAGM medium, blood harvested on CPD externalised a larger percentage of PS throughout 4 weeks. At the end of the 4 weeks period, they had a 8.78 % for the CPD collected blood compared to only 4.65 % for the heparin collected blood (almost double). It should be noted that PS externalization is the first stage, early occurrence of cell death by apoptosis.

Flow cytometric assessment of extracellular pH influence from CPD media on ROS production

Reactive oxygen species (ROS), unstable molecular species possessing an unpaired electron, are created continuously in cell as product of metabolism. Inside erythrocytes, like in all other cells, reactive oxygen species (ROS) are extremely dangerous. They attack proteins or lipoproteins, inducing their damaging. In a grate manner, ROS are triggering factors of apoptosis and cellular death.

Oxidative stress is characterized by decreasing of the intracellular antioxidants (particularly GSH) and free "scavenger" radicals molecules (vitamin E and C), inhibiting some enzymes activity that contribute to metabolism and detoxification of reactive oxygen species like glutathione peroxidase (GPx), GSH reductase, GSH-transferase, catalase (CAT) and superoxide dismutase (SOD), and a consequent increase in ROS production (radical superoxide anion, hydrogen peroxide, peroxy radicals, hydroxyl radical, nitric oxide radical peroxynitrite, etc.). Under these conditions, oxidative stress contributes to the induction and running of apoptosis phenomenon.

Measurement of reactive oxygen species (ROS) is very difficult due to their very short life time and used investigation methods ("electron spin resonance" and "spin trapping") which are complicated and provide approximate values when heterogeneous populations are analyzed. Because ROS have a very short lifetime and current methods are not sufficiently accurate, sensitive and may produce incorrect results especially in heterogeneous cell populations, we tried to determine by flow cytometry due to the multiple advantages of this method. Determination of reactive oxygen species ROS by flow cytometry was tested on numerous cell types and it consists mostly of cases in marking the cells with fluorescent dyes. 2',7'-dichlorofluorescein diacetate (DCFH-DA) is cleaved in a first step into a non-fluorescent product (DCFH) by intracellular esterases and then is oxidized by ROS in a green fluorescent product (DCF) which can therefore be measured by flow cytometry

Based on these data and data from the literature for measuring oxidative stress on numerous cell types, including normal or thalassemic red blood cells, by flow cytometry, the aim of our study was to assess the ROS production in RBCs under the action of extracellular CPD's acidic pH compared to heparin as blood sampling media prior to its preservation in SAGM for 4 weeks.

As shown in figure 3, the amount of ROS within 2 series of blood preservation are significantly different, being much greater for RBCs taken on CPD, even just 16 h after sampling. The amount of ROS becomes maximum after a week of conservation in SAGM media, being double that the one detected in blood collected on heparin (MFI-169.8 compared to 81.64). In the case of blood collected in heparin, the amount of ROS becames maximum with a one week delay, after 14 days of conservation (MFI-87) and diminishing at the end of the period reaching a MFI-63.8. At the end of 4 weeks conservation, ROS production in blood collected on CPD is double (MFI-118.9).

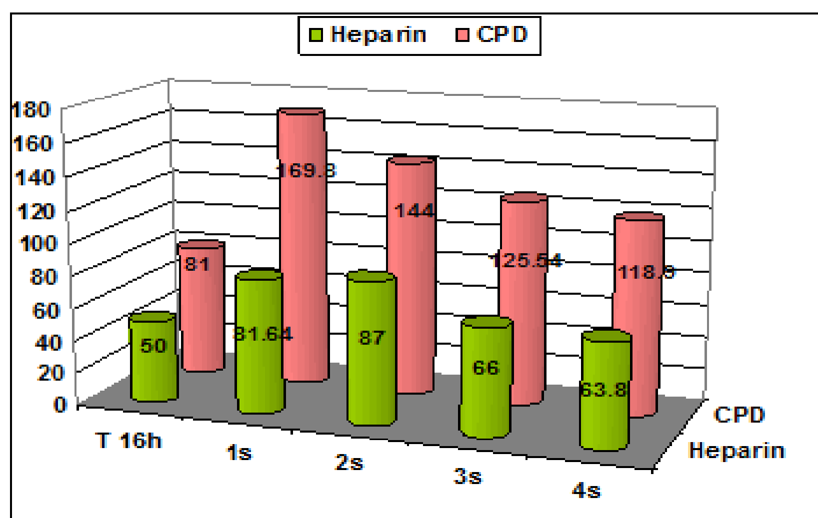

Fig. 3 Comparative histogram of ROS levels under the influence of acidic pH (pH 5.6) of the CPD sampling medium and heparin during storage for four weeks in SAGM medium. The values represent the MFI of the fluorescence. Results are representative of two experiments performed.

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Flow cytometric assessment of the acidic extracellular pH influence from the anticoagulant medium on erythrocytes viability determined with Calcein-AM

Determination of erythrocyte viability is essential in determining the quality of blood for transfusions or in case of haematological pathologies. The major difficulty of determining the viability of erythrocytes is that, (compared to eukaryotic cells where its calculation is made with methods based on vital dyes that bind to the nucleus or mitochondria), human RBCs lack in intracellular organelles, which made long time difficult determining the viability of erythrocytes with the existing methodology.

Since 2005, with the development of a flow cytometry method based on the use of Calcein-AM for determination of the red blood cells viability (Bratosin *et al.*, 2005), this method is used widely internationally. Calcein-AM is the best cell viability indicator due to higher retention in cell and its relative emitted fluorescence insensitivity at physiological limits of pH.The results obtained for the exploration of influence of extracellular pH from anticoagulants medium on cell viability are shown in figure 4.

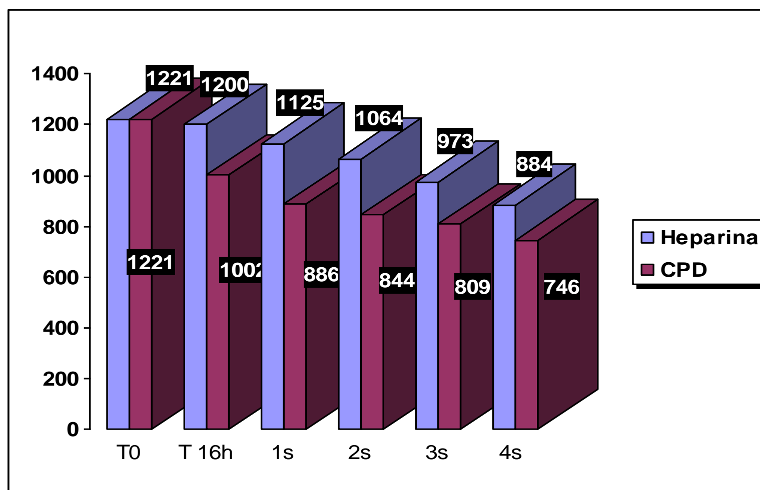

Fig. 4 Comparative RBCs viability under the influence of acidic pH (pH 5.6) of the CPD anticoagulant medium and heparin during storage of RBCs for 4 weeks in SAGM medium. The values represent the average of fluorescence intensity in FL1 (MFI) of fluorescent calcein. Results are representative of two experiments performed.

It is easily observed that during the entirely storage in SAGM medium, blood collected onto heparin retains much better cell viability than the one taken on CPD. Viability of RBCs collected on CPD decrease by 16.5 % until preserving of blood on SAGM medium. This decrease is higher, 21 % for the first week, and in the second week continuing to reduce, by 17 % for week 3 and 16 % for 4th week. Overall, the viability of RBCs taken on CPD is lower by 18 % compared to those taken on heparin.

To conclude, in 2000, Beutler, into an editorial in the Transfusion journal, stated: 1) the fundamental nature of the " storage lesion " remains unknown; 2) no good surrogate test has ever been found for the performance of viability studies in human volunteers; 3) it is unclear which, if any, of the components of the storage systems were effective in improving the viability of stored RBCs after transfusion; and 4) many of the improvements were achieved without the discovery of any new principles (Beutler, 2000).

We suggest that the flow cytometric criteria that we developed could provide that missing window that would permit the direct observation of the changes on the RBCs surface as possible determinants to evaluate the stored erythrocytes viability. While research efforts are directed to better understand the effects of storage on RBCs and the potential impact on transfusion outcomes, the progress is slower in explore ways to limit the negative effects of storage lesion on RBCs.

CONCLUSIONS:

The results of our researches revealed the involvement of an apoptosis phenomenon in accelerated aging of red blood cells in blood bags as the basic phenomenon of storage lesions. Also, our results have validated our new proposed cell viability and apoptosis detection assays by flow cytometry to explore the quality of stored blood components for transfusion after blood storage.

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