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INFLUENCE OF ACIDIC EXTRACELLULAR pH FROM PRESERVATIVE MEDIUM ON INTRACELLULAR pH DURING STORAGE IN SAGM ADDITIVE SOLUTION

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ABSTRACT: Erythrocytes (RBCs) transfusion is a crucial treatment to save lives in case of illness or severe anemia caused by chemotherapy or blood loss due to trauma, accident or major surgery. All currently licensed additive solutions for RBCs have an acidic pH (approx. 5.6-5.8), which is far below the normal physiological pH of 7.3 of venous blood. Because blood sampling is done in an anticoagulant medium with an acidic pH, we aimed to study by flow cytometry, the morphology and physiology changes induced by acid pH. The results of our researches revealed the negative influence of the extracellular acid pH on the much earlier and faster appearance of storage lesions on red blood cells. We have demonstrated the efficiency and sensitivity of flow cytometry methods used in assessing the quality of stored components.

Keywords: RBCs, intracellular pH, storage conditions, blood banks, flow cytometry, scanning electron microscopy

INTRODUCTION:

Erythrocytes (RBCs) transfusion is a crucial treatment to save lives in case of disease or severe anemia caused by chemotherapy or blood loss due to trauma, accident or major surgery.

Before being transfused, red blood cells (RBCs) are stored under controlled conditions for a certain period of time, up to 42 days. For decades, the RBCs components have been prepared as concentrates in additive solution with nutrients that maintain and extend the life of the RBCs component, allowing storage under refrigerated conditions (Hogman *et al*., 2006; Sparrow, 2012).

During storage, blood components are subjected to complex and progressive morphological and biochemical changes called "storage lesions", which may alter their biological functions (Tinmouth *et al*., 2006), representing a major risk factor for increased morbidity and mortality for certain groups of patients. Cold storage of RBC in blood banks has been reported to be associated with changes in various RBCs properties, including cell volume decreasing (Arduini *et al.,* 2007), membrane protein and lipids oxidation (Willekens, 2005), loss of cellular antioxidant capability (Dumaswala *et al.,* 2000), changes in K^+ and Na^+ concentration (Cicha *et al.,* 2000), changes in lipid in/out distribution on the RBC surface (Dumaswala *et al.,* 2000; Bratosin *et al*., 2001) and decreased cell membrane area (Jank *et al*., 2011; Kriebardis *et al*., 2008). RBC storage-induced damage, which can be noticed from the second week of storage and progresses with storage duration (Hess *et al*., 2010), has been shown to impair stored RBCs (St-RBC) functionality (Barshtein *et al*., 2011) and to facilitate their removal from circulation after transfusion (Bosman *et al*., 2008; Bosman *et al.,* 2010; Bosman *et al.,* 2011; Luten *et al.,* 2008). The complexity of the inter-relationship between RBCs biochemistry, cytoskeleton structure and membrane properties were difficult to predict the way how RBCs will respond to different storage conditions.

RBCs exposure to non-physiological base storage media revealed the existence of previously unknown biochemical mechanisms from red blood cells, including similar apoptotic processes, ion and osmotic channels that behaves differently than expected, exposure of new or modified receptors due to oxidation and/or protease/glycosidases activities or senescence (Bratosin *et al*., 1998; Bratosin *et al*., 2001; Bratosin *et al*., 2002; Bosman *et al*., 2008).

In the current conditions of blood transfusion, more than 30% of RBCs are removed from circulation within 24 hours and 75% after 48 hours from transfusion (Mollison, 1984; Luten *et al.*, 2008), raising questions about the efficiency of transfusion and induced negative consequences to politransfused patients. Among storage injuries that occur, reducing ATP levels and hemolysis rates are routinely determined. According to European Council Directive 2004, mandatory minimum general conditions for the transfused red cells require that hemolysis rate should not exceed 0.8% and a proportion of minimum 75% of transfused cells must remain in circulation 24 hours after transfusion (Dumont *et al.,* 2008).

All currently licensed RBCs additive solutions have an acidic pH (approx. 5.6-5.8), which is far below the normal physiological pH of 7.3 of venous blood. Acid additive solutions (and anticoagulants) are used simply because it is easier to heat and sterilize a solution containing glucose at an acidic pH. At the physiological or alkaline pH, glucose is roasted during heat sterilization.

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Because blood sampling is done in an anticoagulant medium citrate-phosphate-dextrose (CPD) pH 5.6, and most conservation medium have also an acidic pH, such as SAGM (Sodium Adenine Glucose Mannitol) pH 5.1 ± 0.3, we aimed assessing the influence of extracellular pH medium on intracellular pH and the induced morphology and physiology changes.

The researches were conducted on two different subjects, and because the results were similar, we have summarized the presentation of a single significantly experiment.

Measurements were made by flow cytometry methods (laser fast, statistical and reproductive technology) respectively: morphology changes analysis in FSC/SSC system (proportional to cell-surface area or size/cell granularity or internal complexity) completed by electron microscopy (SEM) examination.

MATERIALS AND METHODS:

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 (Citrat-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 (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 with a minimum of 10000 cells analyzed in each condition. The results were expressed as MFI value of the logarithmic fluorescence intensity.

Morphological changes analysis by scattered light flow cytometry in FSC/SSC mode

Analysis of the scattered light by flow cytometry in the FSC/SSC mode provides information about cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with the cell size. The intensity of scattered light measured at a right angle to the laser beam (side scatter, SSC), correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light. The cells ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane and, in the case of apoptosis, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies. During apoptosis, the decrease in forward light scatter (which is a result of cell shrinkage) is not initially paralleled by a decrease in side scatter. A transient increase in right angle scatter can be seen during apoptosis in some cell systems. (Darzynkiewicz *et al.*,1997).

Intracellular pH determination

Intracellular pH determination was done according a protocol developed by Franck (Franck *et al*., 1996) for the study of intracellular pH in nucleated cells, based on the following principle: BCECF-AM (2',7'-bis-(2-carboxyethyl)-5,6 carboxyfluorescein acetoxymethyl ester), a cell-permeant, dual-excitation ratiometric pH indicator with a pK_a of ~6.98 is cleaved by intracellular esterases. Intracellular pH measurements with BCECF are made by determining the pH-dependent ratio of emission intensity (detected at 535 nm) when the dye is excited at ~490 nm versus the emission intensity when excited at its isobestic point of ~440 nm. To measure intracellular pH, was set a standard curve, cells were incubated in phosphate buffers of different pH in the presence of nigericin permitting inside and outside pH medium equalization. The pH of the cell was determined compared to the standard curve.

Working protocol: 1 mM nigericin solution was prepared in absolute ethanol (5 mg/6.89 mL), which can be preserved for several weeks at 4 °C. Was prepared a stock solution of 2',7'-bis-(2-carboxyethyl)-5-(and-6) carboxy-fluorescein acetoxymethyl ester (BCECF-AM) 1 mM in DMSO (1 mg/1136 mL) which was kept at – 20 °C in dry medium and protected from light. A and B phosphate buffers, required for obtaining the different pH buffers for calibration (by mixing various volumes), was prepared:

* Buffer A: KH_2PO_4 135 mM (18.37g/L) + NaCl 20mM (1.17g/L)

* Buffer B: K_2HPO_4 110 mM (19.16g/L) + NaCl 20mM (1.17g/L)

Table 1.
Prepared buffers with a preset pH range

pH obtained	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.4
Volume Buffer A	16.60	15.08	13.06	11.40	9.30	7.09	4.80	3.94
Volume Buffer B	3.40	4.92	6.94	8.60	10.70	12.91	15.20	16.06

10^7 RBCs/mL were incubated in PBS with 10 µL BCECF-AM 1mM for 30-60 min, at 37 °C (10 µM final concentration of BCECF).

A calibration curve was prepared for each series of analyzes as follows: 100 µL cells are incubated with BCECF, added over 10 µL extemporaneously diluted nigericin (1 µM final) and 900 µL phosphate buffer at the desired pH (from pH 6.0 to pH 7.4). Cellular suspension was incubated at 37 °C for 10 minutes and then immediately placed on ice, coloration being stable for 2 hours. 20000 cells were cytofluorimetric analyzed for green fluorescence FL1 (BCECF) linearly and FL2 for red fluorescence (BCECF), also in a linear mode.

Scanning electron microscopy analysis (SEM)

Scanning electron microscopy analysis (SEM) is a fine investigating method of cell surfaces resulting in a three-dimensional representation.

Red blood cells were fixed for 4 h with 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and then washed 3 times with distilled water and finally filtered on 0.2 µm filters (Anodisc filters). After drying, the sample was visualized on a scanning electron microscope Hitachi SU 1510.

RESULTS AND DISCUSSION:

Effect of extracellular acidic pH of CPD anticoagulant on the whole blood collected

In the first stage we studied the effect of acid pH of CPD medium on human red blood cells from collected blood, by FSC/SSC flow cytometry.

In figure 1 is easily seen that erythrocyte distribution at the collected time (6h after sampling) on CPD (Fig.1B) compared with normal erythrocytes (Fig.1A) had suffered morphology modifications consisting in both, size and density changes. After one week was observed apparition of a great number of ghosts, (results of erythrocytes hemolysis) that are placed in the area marked by the arrow (Fig. 1C).

Analysis of the extracellular pH of the CPD collected medium indicated the change of pH from pH 5.6 to pH 6.8 after 6 hours, reaching a neutral pH of 7.6 after 1 week. This change is due on the one hand to the buffer capacity of blood plasma and also to ion exchange at RBCs levels as indicated by SSC cytograms change. These biochemical processes inevitably lead to a disorder of cellular content and hence the physiology of erythrocytes.

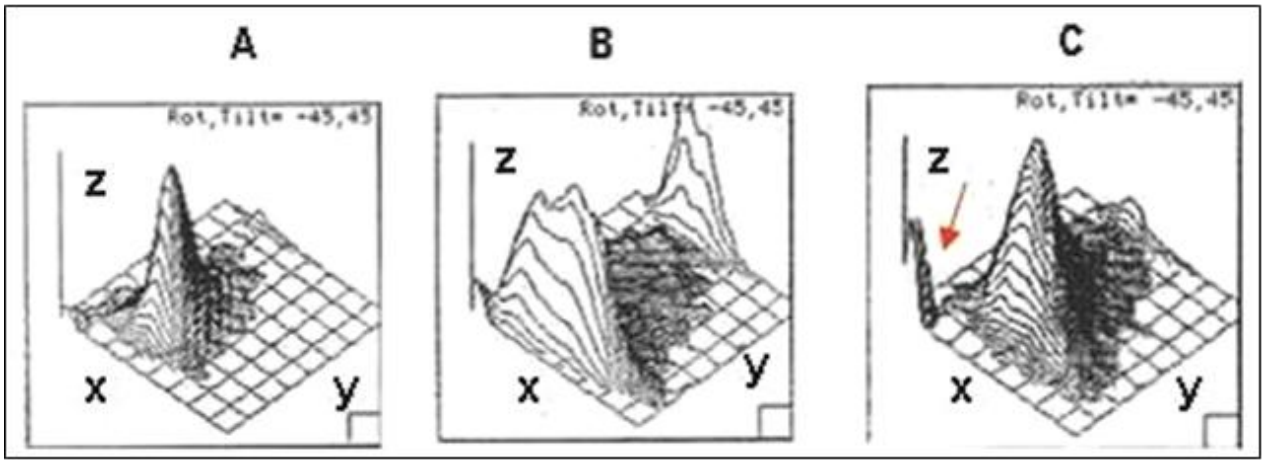

Fig. 1 Flow cytometry analysis in FSC/SSC system (cell waist/cell content) of the acid pH influence (pH 5.6) of CPD sampling medium. A-To; B-6h after sampling; C-after 1 week; x-FSC; y-SSC; z-cells number; arrow indicates the area of cellular debris (ghosts)

Direct assessment by scanning electron microscopy analysis (SEM) of morphological change of RBCs induced by extracellular pH of acidic CPD during storage in SAGM additive solution

Due to results of first experiment, we compared the behavior and modifications of RBCs from the same donor collected on anticoagulant CPD and heparin.

Subsequently, erythrocytes were preserved in both cases in MacoPharma mini-bags with SAGM medium and weekly analyzed for four weeks by flow cytometry for morphology changes, intracellular pH measurement and cell viability.

Red blood cells from the same donor, collected on heparin or CPD were left for 16 hours at 20 °C according to the protocol from transfusion centers. Then, RBCs were transferred to the SAGM mini-storage bags, with 60% hematocrit in both cases and then preserved at 4 °C.

Scanning microscopic analysis of RBCs morphology prior to storage in SAGM additive solution is shown in figure 2.

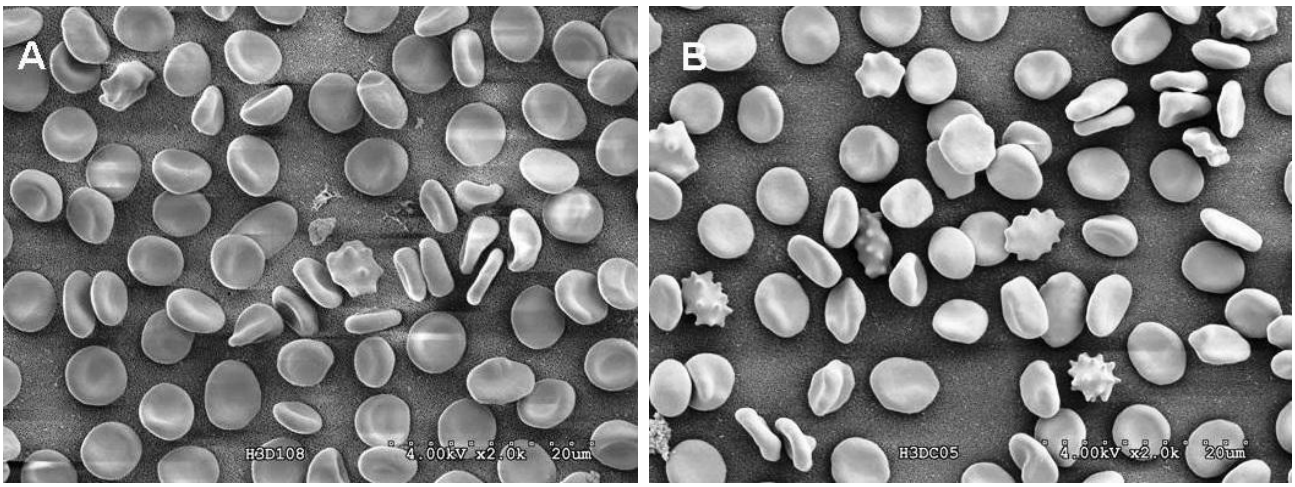

Fig. 2 Scanning electron microscopic analysis of blood collected on heparin (A) and on CPD medium, pH 5.6 according to the practices from transfusion centers (B) after 16 h at 20°C, before passage into the SAGM additive solution

From figure 2, in the case of RBCs collected on CPD medium, pH 5.6, after 16 hours, noted the appearance of a large number of echinocytes.

Assessment by direct FSC/SSC flow cytometry of extracellular pH influence from anticoagulant preservative on red blood cells morphology during SAGM storage medium

The parameters measured by flow cytometry are optical signals whose intensity can be correlated with cell properties. Assessment of cell morphology by measuring the absorption and diffusion of light is also known as "direct analysis in FSS/SSC system" (light scatter measurements).

After intercepting the incident light, cell emit certain signals. Light scattered at an acute angle or shaft (FSC) can be correlated with cell size, enabling to distinguish a cell from aggregates or cellular debris and assessing cell viability, because dead cells diffuses weaker the light in this direction. Light scatter (SSC) that diffused under a right angle allows the study of refractive cytoplasmic of cell content. The analysis of a cell population based on two FSC/SSC analyzed parameters generates a cytogram or a "dot-plot", where each cell is represented by its identified values by a point. Cells with characteristics similar to the two analyzed parameters represent a cell population or subpopulation and presents itself as a "cloud", allowing discrimination of living cells to the apoptotic or dead, because dead cells diffuses light much less in FSC.

From both figures, figure 3 and figure 4, it was observed a better preservation of erythrocyte morphology collected on heparin by XGeoMean values that are much closer to the original, while erythrocytes collected on CPD decrease cellular size was manifested yet the first 16h, to be more decreased after 4 weeks in medium SAGM. More important are changes in cellular content expressed by YGeoMean values that diminish greatly if erythrocytes was collected on CPD, which means significant changes of intracellular composition.

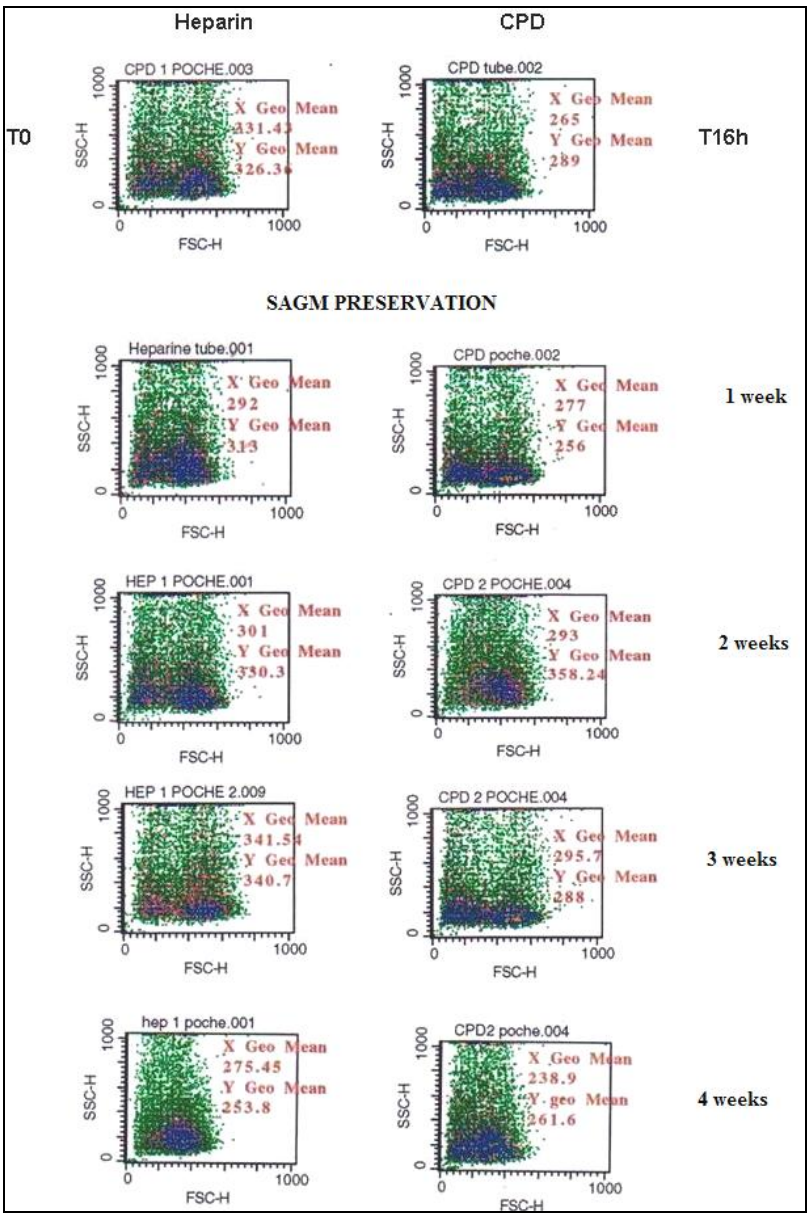

Fig. 3 Flow cytometric analysis FSC/SSC of the influence of CPD preservation medium, pH (pH 5.6) on cell morphology. XGeoMean-cell size average value; YGeoMean-cellular content average value. The number of cells analyzed -10000. The results shown are representative of three experiments performed

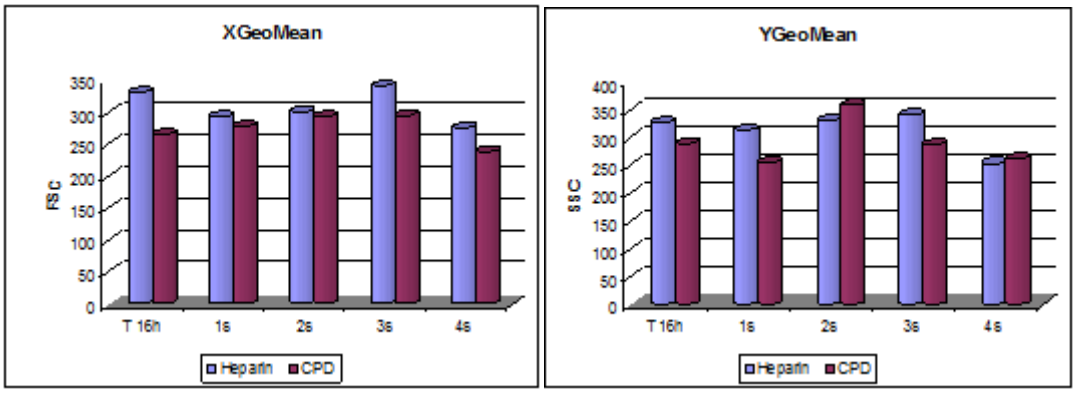

Fig. 4 Comparative flow cytometry analysis of X and Y GeoMean values for RBCs preserved in SAGM medium for 4 weeks after collecting on heparin and CPD. XgeoMean-cell waist average value; YGeoMean-cellular content average value. The number of cells analyzed-10000. The results shown are representative of three experiments performed

Flow cytometric assessment of the extracellular pH influence on the RBCs intracellular pH

As can be seen in figure 5 and 6, from comparative histogram of intracellular pH evaluated by flow cytometry under the influence of acid pH (pH 5.6) from CPD medium during storage for four weeks in SAGM medium, were not observed major changes in intracellular pH after the two series of donors, the difference being of maximum 0.4 units for analyzes conducted to every time of conservation. In all cases, the intracellular pH was not below pH 7.0.

To be noted that the sensitivity of the intracellular pH determination by flow cytometry is very high, and the measurement was made in triplicate.

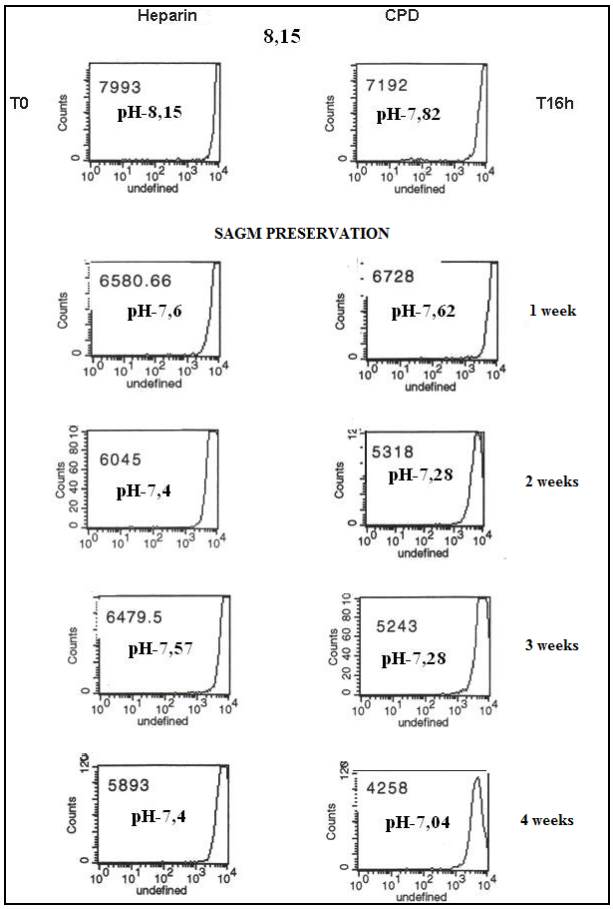

Fig. 5 Flow cytometric analysis of intracellular pH under the influence of acid pH (pH 5.6) of CPD prelevation medium and heparin during storage for four weeks on SAGM medium. The number of cells analyzed were 10000. Results are representative for two donors and three experiments performed

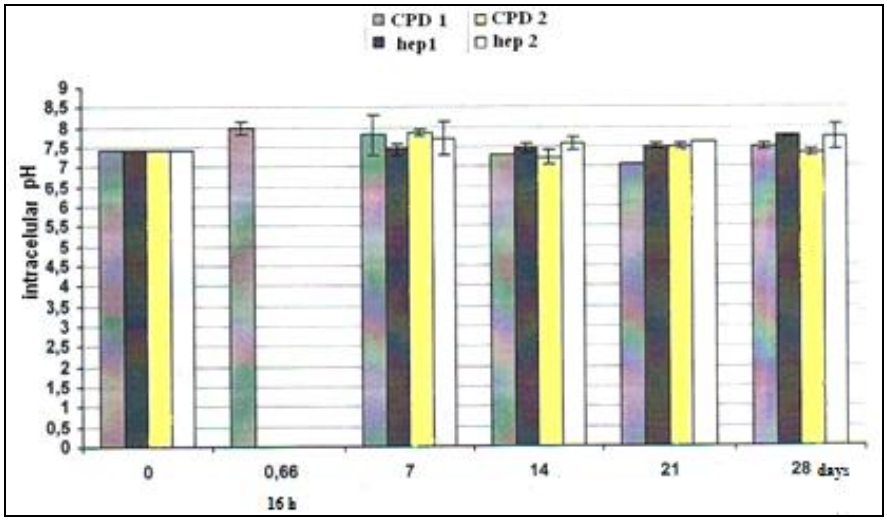

Fig. 6 Comparative histogram of intracellular pH evaluated by flow cytometry under the influence of acid pH (pH 5.6) of CPD prelevation medium and heparin during storage for four weeks on SAGM medium. The results shown are representative of two experiments made, each determination being performed in triplicate

pH effects on red blood cell's metabolism are very complex. Intracellular acid environment alters the activity of certain enzymes and biochemical pathways. For RBCs, intracellular acidification interferes with the production of adenosine 5'-triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG), which are essential for the survival and RBCs function in oxygen providing. During RBCs storage in acid additive solutions, the intracellular concentration of ATP and 2,3-DPG declines (van der Meer *et al.,* 2011). In 1996, Gros and collaborators (Gros *et al*., 1996) concluded that shape changes and vesiculation were induced in intact human erythrocytes by gradually decreasing pH in cell suspension.

In the last 15-20 years, researches into developing new additives solutions was focused on maintaining a higher level of intracellular ATP and 2,3-DPG during RBCs components storage. Although the wrong misconception that ATP levels in cells determines whether cells survive or not, this is far from being a reliable indicator of viability. It is true that red blood cells with low levels of ATP can not phosphorylate glucose and therefore are doomed to die, but high levels of ATP do not ensure the survival of stored red cells (Sparrow, 2012).

Particularly important is the effect of external pH of additive solutions on erythrocyte. Standard preservatives, ACD (citrate-dextrose) and CPD (citrate-phosphate-dextrose) solutions have an acidic pH of 5.0 respectively 5.6. When erythrocytes stored in ACD medium were examined *in vivo* by viability studies, the investigators found that it was superior to glucose-sodium citrate preservative, which was previously used. A common misunderstanding is that these preservatives acidify the blood. These beliefs are based on measuring the pH of blood stored at 37 ºC, although the storage temperature is 4 ºC. Temperature has a profound effect on the pH of the blood. Cooling blood from 37 ºC to 4 ºC increase external pH between 0.4 and 0.5 pH units causing a higher change of internal pH. These, along with decreasing the concentration of hydrogen ions have been encountered in the use of ACD and CPD solutions so that the pH of the extracellular blood collected anaerobically at 4 ºC is about 7.5 to 7.7, and the intracellular pH is 7.6 and 7.8. The reversal of the usual pH gradient between the interior and exterior of the cell is probably caused by the Donnan effect exerted by the citrate ion in the medium. They were barely visible in stored blood with ACD and CPD at 37 ºC and 4 ºC.

The hydrogen ion concentration does not remain fixed during the storage of blood. While red blood cells metabolize glucose, they produced huge quantities of lactic acid. For example, after 21 days of storage in CPD solution, lactate concentration of stored blood increases about 15 mM, the hydrogen ion concentration is one of the factors that influence the metabolism of red blood cells.

Also is known that pH has influence on ATP erythrocytes level. ACD pH (pH=5) seems to be optimal for long-term preservation of ATP from RBCs (Beutler *et al*., 1965). If adenine was added on ACD, the pH became 5.5 (Beutler *et al*., 1965). When blood is collected in a preservative medium at a level of pH near physiological external pH, it is reached a slightly larger internal physiological pH value. When the initial pH of the preservative is less than 5.0, the preservation of ATP in RBCs is satisfactory for 1-2 weeks. While lactic acid accumulates, pH suffocates glycolysis by inhibiting HK and PFK and deprive RBCs source of energy. Consequently, ATP level drops quickly. If the pH level of the environment is too high, then the events are different. ATP lowering of RBCs is acute and is observed from the early hours. Within 24 hours, the ATP may decrease by 50% or more, compared to baseline. The reason for this remarkable ATP loss of RBCs is more subtle than the loss observed with low pH levels. Rapid depletion of ATP in the case of preservative with high levels of pH, requires presence of glucose (Beutler *et al*., 1965). The storage of blood in the ACD pH 7.0 results in rapid decline in ATP levels in the absence of pyruvate, but, the addition of pyruvate which oxidate NADH to NAD prevents this loss.

Adding phosphate ions exerted the greatest buffer effect on the pH level. Moreover, when adenine is present in preservative medium, phosphate strongly stimulates the formation of ATP. The amount of phosphate present in the CPD solution is too small to affect the pH from blood and preservative mix. When using higher amounts, the phosphate effect slows down the pH lowering. Phosphate strongly stimulates glycolysis. Thus, while acting as an effective buffer for lactic acid produced during glycolysis, at the same time increase its production. This is probably why the use of high levels of phosphate in preservatives has little practical value. Large amounts of ATP that results are not associated with improved viability, and lactate production increase compensates for the increased buffer capacity and the preservation of 2,3-DPG is not fully performed (Beutler *et al*., 2000).

In conclusion, the RBCs have a sufficient buffering capacity to adjust the pH close to physiological levels during the first few days of storage in an acidic medium. However, the buffering capacity of red blood cells is quickly exhausted by the generation of lactic acid by red blood cells via anaerobic glycolytic pathway. As a consequence, RBCs extracellular and intracellular pH becomes progressively more acidic during storage, reaching a pH of 6.5 after 6 weeks of storage in additive acid solutions (van der Meer *et al*., 2011).

CONCLUSIONS:

The results of our research revealed the negative influence of the extracellular acid pH on the much earlier and faster appearance of storage lesions on red blood cells, on a comparative manner on blood prelevated in conditions of transfusion centers, respectively CPD pH 5.6 and heparin. In the same time, we have demonstrated the efficiency and sensitivity of flow cytometry methods used in assessing the quality of blood stored components.

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