STUDYING THE AGING OF BANKED ERYTHROCYTES USING A FLUORESCENCE MARKER, HYPERICIN

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Abstract. Banked blood used for transfusions can be banked for 42 days, but there are several issues that can make it unusable: biochemical and biomechanical changes that leads to storage lesions of erythrocytes. These changes can cause multiple health problems for patients who receive 42 day banked blood. The aim of the project was to develop an efficient method for determining the quality of banked erythrocytes using hypericin as a fluorescent marker for assessing the erythrocyte membrane integrity. We made biochemical determinations for the oxidative stress, antioxidant capacity and energy metabolism. Concomitantly, we monitored hypericin fluorescence added to erythrocytes with the aim to highlight their membrane changes occurring during the storage period. The results that we obtained indicate that erythrocytes suffer a strong oxidative stress during storage, correlated with a decrease of antioxidant capacity, in association with alteration of their membranes. Our data suggest that hypericin can be used as a fluorescent marker for determining the quality of banked blood.

INTRODUCTION

Erythrocytes are 7 μm diameter blood cells; their membrane includes proteins with structural, antigenic, enzymatic and transport functions. 90% of red blood cells weight is represented by hemoglobin. The erythrocyte has the following functions: transport of O_2 and CO_2, and in hypoxia conditions the hematite produces nitric oxide involved in regulation of microcirculation (Almac *et al*., 2007). Blood used for transfusions can be stored for 42 days, and the acceptable 24-hour *in vivo* survival of RBCs after transfusion has been defined as 75% (Walker *et al*., 1993). A variety of changes have been identified within the red blood cell (RBC) and storage media during RBC preservation that are correlated with reduced tissue oxygenation and transfusion-associated adverse effects. These alterations are collectively termed as “storage lesions” and include extensive biochemical, biomechanical, and immunologic changes. These changes can cause multiple health problems for patients who receive 42 day banked blood; transfusion of older blood significantly increased the risk of complications following coronary artery bypass and/or heart-valve surgery (Koch *et al*., 2008). Experimental studies indicate that aged RBCs lose their deformability and adhere to endothelial surface, causing capillary occlusion (Hovav *et al*., 1999). Many studies, using a variety of methods including filtration, micropipette elongation, and laser-assisted ektacytometry, have shown that the deformability of RBCs decreases progressively during storage (Berezina *et al*., 2002; Haradin *et al*., 1969; d’Almeida *et al*., 2000; Izzo *et al*., 1999; Card *et al*., 1983, 1982). Such measurements suggest that membrane loss is responsible for decreased deformability over time during storage (Izzo *et al.*, 1999; Card *et al.*, 1982). Loss of RBC membrane integrity and reduced red cell deformability, as occurs with RBC storage, has raised concern over the potential for microcirculatory occlusion and resultant tissue ischemia (Marik *et al*., 1993). Conceptually, the lungs are particularly susceptible to the adverse effects of banked RBCs as the pulmonary microcirculation is the first exposed to the mediators of a storage lesion. A particularly strong correlation was noted between mean duration of RBC storage and postoperative acute kidney injury. In addition to the adverse effects described above, RBC transfusion has also been associated with the development of multi-organ failure (Zallen *et al*., 1999; Moore *et al*., 1997). Chief among these concerns is the mounting evidence correlating RBC transfusion with risk-adjusted mortality (Purdy *et al*., 1997; Leal-Noval *et al*., 2003). Importantly, multiple studies have suggested this association becomes stronger with increasing duration of RBC storage (Kor *et al*., 2009).

The aim of our study was to develop an efficient method for determining the quality of banked erythrocytes using hypericin as a fluorescent marker for assessing the erythrocyte membrane integrity. We made biochemical determinations for the oxidative stress (carbonyl content and malondialdehyde – MDA), antioxidant capacity (superoxide dismutase – SOD, catalase – CAT, glutathione peroxidase – GPx, glutathione reductase – GRe and thiol groups – SH), energy metabolism (pyruvate and lactate dehydrogenase – LDH) and cholesterol content from three distinct donators. Concomitantly, we monitored hypericin fluorescence added to erythrocytes with the aim to highlight their membrane changes occurring during the storage period. After that, we correlated the biochemical results with the imagistic ones.

MATERIALS AND METHODS

Blood samples. In our study we used erythrocytes taken from three distinct donors and banked in PVC bags with CPDA anticoagulant solution. Erythrocytes were kept at a temperature of 4° C for 49 days. Biochemical and imaging studies were performed on blood samples taken from storage bags with an interval of seven days: day of storage 1, 7, 14, 21, 28, 35, 42 and 49. Erythrocytes were counted in every sample analyzed with blood component analyzer Nikon Cell Tech for a better quantification of values obtained from tests carried out. To keep their biochemical and morphological characteristics of the time of sampling until their analysis RBCs were cryogenated at -80° C (for the first time in Romania) as previously described (Valeri *et al.*, 2000; Han *et al.*, 2005). Thawing was achieved by water bath at a temperature of 37° C for 2 minutes, followed by centrifugation at 5000 RPM (D-78 532 Hettich centrifuge) for 10 minutes at 25° C; further, erythrocytes were washed with 12% NaCl solution to remove the remained glycerol. After the process of thawing, erythrocytes were recovered in a proportion of 75% (Fig. 1), in agreement with literature data (Meryman, 1989; Valeri *et al.*, 2000; Han *et al.*, 2005).

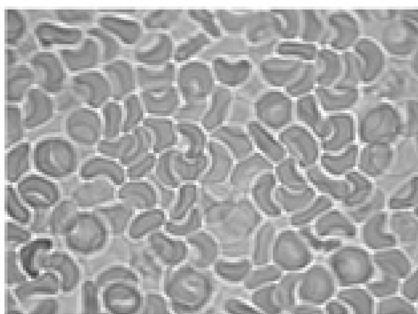

Fig. 1. Erythrocytes after thawing. The membrane of erythrocytes kept its shape and integrity completely.

Imagistics. Samples used for confocal microscopy contained 5 μl + 5 μl hypericin solution. Excitation of hypericin was obtained at 480 nm and the fluorescence emission was obtained at 550-600 nm. The concentration of hypericin was 1.01 x 10^{-2} μmols/ml. We used Nikon AR confocal microscope and NIS Ellements AR data software.

Preparation of hypericin. Hypericin used for fluorescent marking of RBC membranes was obtained from Hipericum perforatum L.: over 2 g of chopped plant we added 70 ml petroleum ether and boiled the mixture at 65° C for 2 hours; the ether was removed and the plant was dried, and washed with 30 ml chloroform; the plant was, than, filtered and dried for 10-15 minutes; an addition of 30 ml of 10% acetone over the plant was made, followed by sonication for 30 minutes and filtration. Further, a mixture of ethyl ether and water (1:1) was added over hypericin solution (equal volumes); after a vortex and phase separation we selected the etheric phase (highest concentration of hypericin). Hypericin was characterized by HPLC using a Jasco system: controller LC-Net II /ADC, PU-2089 Plus Quaternary Gradient pump, FP-2020 Plus fluorescence detector, UV-Vis MD-2015 Plus Multiwavelength detector, Teknokroma TR - 015519 chromatographic column (with 5μm 100 C18 particles).

Non-enzymatic assays. *Protein concentration* was determined by the method of Lowry *et al.* (1951) using BSA as standard (Pandey *et al.* 2010). We used 0,2 ml hemolyzate from each sample, with a dilution of 1000 *X*.

MDA. Erythrocyte MDA concentration was determined by the tiobarbituric acid (TBA) assay as previously described (Gutteridge, 1982; Dumaswala *et al.*, 1999); the pink chromogen produced by the reaction of TBA with MDA was measured at 532 nm. We used 0,5 ml erythrocytes of a 30% concentration from each sample.

Carbonyl groups. Erythrocyte carbonyl content was measured according to procedure of Levine *et al.* (1990) and described by Pandey *et al.* (2010); we used 1 ml erythrocytes of a 10% concentration for each sample.

SH groups. The concentration of erythrocyte thiol groups was determined by Ellman method, modified by Albini *et al*., 1980. We used 0,15 ml hemolyzate from each sample.

Cholesterol. We determined the concentration of total cholesterol in banked erythrocytes by Lieberman-Burchard method: over 0,1 ml erythrocytes from each sample we added 0,6 ml sulfosalicylic acid and 1,5 ml acetic anhydride. We added 0,2 ml H_2SO_4, and incubated the samples for 10 minutes in the dark and measured the absorbance at 610 nm (Campbell *et al*., 2005).

Pyruvate. For determining the concentration of pyruvate in banked erythrocytes we used an adapted method of Schwimmerand and Weston (1961): over 0,1 ml hemolyzate we added 0,5 ml 1,26 mM DNPH followed by incubation at room temperature for 20 minutes; further, we added 5 ml 0,4 N NaOH and measured the absorbance at 500 nm.

All the above non-enzymatic assays spectra measurements were made with a PerkinElmer Lambda25 spectrophotometer.

Enzymatic assays.*CAT* activity was determined according to Beers and Sizer (1952). The enzymatic activity was determined by following spectrophotometrically at 240 nm the decomposition of H_2O_2 for 1 minute at 25° C, in phosphate buffer pH 7. We used 100 *X* diluted hemolyzate from each blood sample and a Jasco V-530 spectrophotometer. Specific activity was expressed as units of enzymatic activity per mg of protein.

SOD activity was determined according to Paoletti (1986). The method is based on the capacity of SOD to catalyze the transformation of superoxide anions into molecular oxygen and hydrogen peroxide. The method consists in a reaction sequence in which is generated superoxide anions from molecular oxygen, in the presence of EDTA, $MnCl_2$ and β-mercaptoethanol. The speed of the enzymatic reaction is determined by the decreasing of absorbance at 340 nm as a result of NADPH oxidation, which occludes at 340 nm. For the determination of absorbance of samples we used a TECAN GENios multireader. Before enzymatic determination we precipitated the hemoglobin of erythrocytes according to Sun *et al*. (1988). Specific activity was expressed as units of enzymatic activity per mg of protein.

GRe activity was spectrophotometrically determined as previously described (Mavis and Stellwagen, 1968). A unit of activity is the amount of enzyme that catalyzes the oxidation of 1 μmole of NADPH per min. Specific activity was expressed as units of enzymatic activity per mg of protein. The speed of enzymatic reaction is determined by the decrease of absorbance at 340 nm as a result of NADPH oxidation, which occludes at 340 nm. We used a 3 *X* diluted hemolyzate from each sample. The spectrophotometric measurements was made with a PerkinElmer Lambda25, at 340 nm and 25° C.

GPx activity was measured the modified method of Paglia and Valentine (1967) as described by Jacobson et al. (1982), using tert-butyl hydroperoxide (tBHP) as substrate. We observed the formation of GSSG, which is continuously reduced by glutathione reductase. The determination of GSSG was made by spectrophotometric measurement at 340 nm of oxidized NADPH, at 25° C for 5 minutes after the addition of tBHP. We used a PerkinElmer Lambda25 spectrophotometer. Specific activity was expressed as units of enzymatic activity per mg of protein.

LDH activity was determined according to Korzeniewski *et al*., 1983, using a PerkinElmer Lambda25 spectrophotometer. We followed the decreasing of absorbance at 340 nm for 5 minutes. Specific activity was expressed as units of enzymatic activity per mg of protein.

RESULTS AND DISCUSSIONS

Banked erythrocytes alteration

Human RBCs stored in standard banking conditions undergo time-dependent changes, including loss of ATP, GSH, lipids, and structural and membrane proteins, possibly leading to decreased deformability and unsatisfactory post-transfusion in vivo survival (Dumaswala *et al*., 1997; 1994; 1996; Wagner *et al*., 1987; Geldwerth *et al*., 1993). Reactive species of oxygen (RSO) can modify protein amino acid residues like lysine, arginine, proline and histidine, generating carbonyl content. This could be considered as an early marker of protein oxidation (Levine *et al*., 1990). Considering protein oxidation as a marker for oxidative stress can represent an advantage, comparing to other measurements, because of its early formation and stability of oxidized proteins; more, it can emphasize the severe cases of oxidation (Dalle-Donne *et al*., 2003). The result of protein oxidation is their fragmentation and aggregation, and also loss of functionality (Butterfield and Kanski, 2001; Cabiscol and Levine, 1995; Dalle-Donne *et al*., 2003). This can lead to destabilization of cell metabolism. Several reports have documented that *in vitro* exposure to oxidants increases RBC membrane instability by damaging protein band 4.1 and forming a defective spectrin-band 4.1-actin tertiary complex (Advani *et al*., 1992; Shinar *et al*., 1987; Dumaswala *et al*., 1999). Band 4.1 protein is very important because it promotes a high affinity association between spectrin and F-actin, and it may link the skeleton to the membrane by virtue of its association with glycophorine and band 3 (Dumaswala *et al*., 1999). RBCs lose significant amount of bands 3 and 4.1 proteins during blood banking, and their membranes maintain integrity by releasing damaged segments of the membrane in the form of vesicles (Dumaswala *et al*., 1996). The alteration of these important proteins can negatively affect the architecture and the function of erythrocyte membrane, leading to general destabilization of cytoskeleton and to compromised erythrocyte survival (Kriebardis. *et al*., 2006).

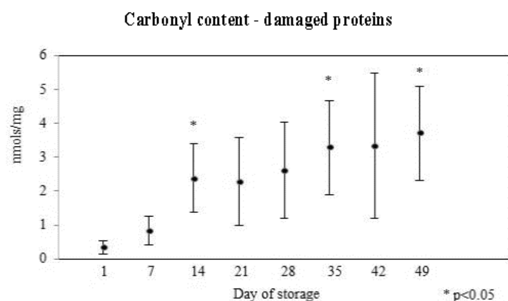

Fig. 2. The evolution of total erythrocyte carbonyl concentration during 49 days of banking. The values are expressed as nanomols/mg of protein and are related to an average concentration of 7.193.333 RBCs/µl, thus, indicating the protein oxidation status. Tests were always made in triplicate for RBCs from each of the three donators. Statistical analysis was made with Student's t-test. The large error variations may be due to the specificity of donators (age, sex, lifestyle etc.).

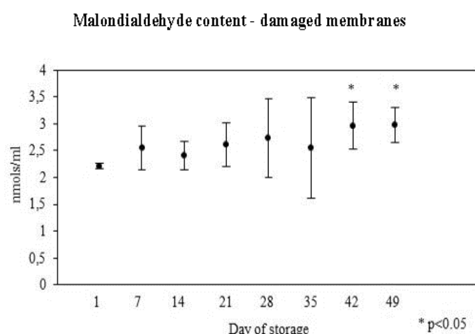

Fig. 3. The evolution of total erythrocyte MDA concentration during 49 days of banking. The values are expressed as nanomols/ml of RBCs and are related to an average concentration of 7.193.333 RBCs/µl, thus, indicating the total lipid peroxidation status. Tests were always made in triplicate for RBCs from each of the three donators. Statistical analysis was made with Student's t-test. The large error variations may be due to the specificity of donators (age, sex, lifestyle etc.).

Oxidative stress also generates MDA. This kind of reactive molecule is involved in the mechanical and functional alteration of plasmatic membrane (Corry *et al.*, 1980; Rice-Evans *et al.*, 1981; Hochstein *et al.*, 1981; Jain *et al.*, 1980; Shinitzky *et al.*, 1978), and represents a marker for lipid peroxidation (Marjani *et al.*, 2007) because it's a final product of the alteration of erythrocyte membrane (Dumaswala *et al.*, 1999). MDA relocates membrane phospholipids and proteins while alters a series of membrane functions. These destructive effects of MDA lead to a decrease of erythrocyte survival, during banking (Jain and Hochstein, 1980; Scott *et al.*, 1989; Hebbel *et al.*, 1990; Sughihara *et al.*, 1991). Also, MDA has been shown to cross-link erythrocyte phospholipids and proteins to impair a variety of the membrane-related functions, and ultimately leading to diminished RBC survival (Jain *et al.*, 1980; Chiu *et al.*, 1989; Hebbel *et al.*, 1990; Sugihara *et al.*, 1991).

In agreement with previous assertions, we observed by imaging studies that erythrocyte membranes suffered a strong and progressive alteration, including deformation, vesiculation, excessive permeabilization and fragmentation during storage (fig. 12). Imagistic data was in strong correlation with the biochemical data that we obtained, regarding erythrocyte MDA and carbonyl content evolution. Our results reported a significant time-dependent increase of protein oxidation level at banked erythrocytes. The protein carbonyl content, also, increased from the first to the last day of storage. The most important increase, of approx. 6-fold, was between days of storage 1 and 14. The erythrocyte carbonyl content in the 49th day of storage was approx. 61% more than in the 14th day (fig. 2). Our results are in correlation with those of Kriebardis *et al.*, (2006), who analyzed the evolution of membrane protein oxidation at erythrocytes banked in CPDA. According to them, the maximum level of carbonyl content was achieved after 28-30 days of storage, and the most significant increase was observed after 5-10 days of storage, similar to our study. Our results regarding the evolution of erythrocyte malondialdehyde content indicated a significant increase of MDA during banking, of approx. 35% in 49 days (fig. 3). These are in correlation with Dumaswala *et al.*, (1999) and Aslan *et al.*, (1997), who reported significant increases of erythrocyte MDA concentration during storage.

Thus, our results regarding carbonyl content and MDA showed a significant increase of protein oxidation and lipid peroxidation at erythrocyte banked for 49 days. This suggests that erythrocytes suffered a continuous general alteration. This could generate destabilization of erythrocyte cytoskeleton (Kriebardis *et al.*, 2006), loss of initial cell shape, alteration of plasmatic membrane (Dumaswala *et* al., 1999; 1996) and a reduced survival of RBCs (Kriebardis *et al.*, 2006). All these degradative morphological phenomena were highlighted by our imaging studies with hypericin. We, also, observed that erythrocyte deformation has increased in correlation with time-dependent carbonyl content and MDA concentration changes.

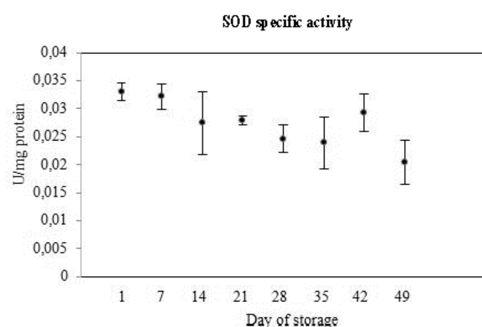

Fig. 4. The evolution of SOD enzymatic activity at RBCs banked for 49 days in CPDA. The values are expressed as U of enzyme/mg of protein and are related to an average concentration of 7.193.333 RBCs/μl. Tests were always made in triplicate for RBC hemolyzate from each of the three donators. The large error variations may be due to the specificity of donators (age, sex, lifestyle etc.).

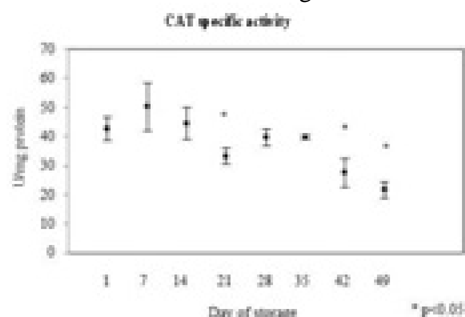

Fig. 5. The evolution of CAT enzymatic activity at RBCs banked for 49 days in CPDA. The values are expressed as U of enzyme/mg of protein and are related to an average concentration of 7.193.333 RBCs/μl. Tests were made in triplicate for RBC hemolyzate from each of the three donators. Statistical analysis was made with Student's t-test. The error variations may be due to the specificity of donators (age, sex, lifestyle etc).

RBC antioxidant system

In humans, the antioxidant system includes a number of antioxidant enzymes such as SOD, CAT, GPx, GRe, and non-enzymatic antioxidants such as GSH and protein–SH (thiol groups). SOD, CAT and GPx are three main enzymatic systems of the organism against free radicals and peroxides (Bogdanska *et al.*, 2003). SOD plays an important role in the protection of cells against the deleterious effect of free radicals by converting superoxide anions to hydrogen peroxide, which is then transformed to water by GPx or by CAT (Ross *et al.*, 2000). In erythrocytes, catalase and glutathione peroxidase jointly protect hemoglobin from oxidative damage (Kisadere *et al.*, 1997). CAT catalyzes the rapid decomposition of hydrogen peroxide by two types of reactions. Both types include a first step formation of compound I, which consists of the enzyme and hydrogen peroxide. The catalytic activity catalyzes a reaction with a second molecule of hydrogen peroxide producing water and oxygen (Johansson *et al.*, 1988; Lardinois *et al.*, 1996). GPx catalyzes the reduction of hydrogen peroxide by GSH (Awasthi *et al.*, 1975), and protects hemoglobin from oxidative breakdown (Mills, 1957). GPx and GSH appear to provide the primary antioxidant defense in banked RBC, and their decline, concurrent with an increase in oxidative modifications of membrane lipids and proteins, may destabilize the membrane skeleton, thereby compromising RBC survival (Dumaswala *et al.*, 1999). GPx catalyzes the reduction of lipid peroxides (Mills, 1957). Thereby, it has been suggested that GPx is capable to break the autocatalytic chain reaction of lipid peroxidation, protecting the plasmatic membrane against oxidative degradation (Mills *et al.*, 1957). GRe is the enzyme which is required to maintain GSH in the reduced state (Beutler and Yeh, 1963). The thiol groups are very reactive to a great variety of oxidant agents (Means and Feeney, 1971). Also, SH groups confer stability to erythrocyte membrane, plying an important role in the molecular arrangement of lipid bilayer

(Haest *et al*., 1983). Critical changes that affect the erythrocyte lifespan occur at their membrane SH groups (Parker and Hoffman, 1964). The thiol groups include free GSH which is a very important factor in erythrocyte antioxidant system. GSH combines with different types of endogenic and exogenic compounds, and it is cofactor for different enzymes; erythrocytes contain lot of GSH. Thereby, the fall of thiol groups concentration suggests that the antioxidant defense of erythrocytes is decreasing and the plasmatic membrane is unstabilizing, during banking.

In our study we observed that the specific activity of SOD significantly decreased; in the 49th day of storage enzymatic activity was approx. 37% less than in the first day of storage (fig. 4). Like SOD, CAT showed a decrease of approx. 50% of its specific activity during storage period. We also observed that enzymatic activity of SOD begun to decrease from day of storage 7 (fig. 5). Likewise, we observed a constant decrease, of approx. 24%, of GPx enzymatic activity, during banking (fig. 6). The most significant fall of GPx activity was observed after day of storage 21. The enzymatic activity of GRe presented the same decreasing trend; the most evident decrease of GRe activity was noticed between day 7 and day 42; the difference was approx. of 27% (fig. 7).

Our result indicated a decrease of approx. 40% of SH groups concentration in banked erythrocytes (fig. 8); a reason for this could be the increase of protein oxidation (fig. 2); another reason could be the consumption of glutathione in detoxifying reactions. Between day 1 and day 21 we observed a decrease of approx. 38% of SH groups concentration, followed by an increase of approx. 18%, until day 28, and another decrease of approx. 17% until day 49. So, the most important decrease of SH groups concentration was recorded between day of storage 1 and day 21.

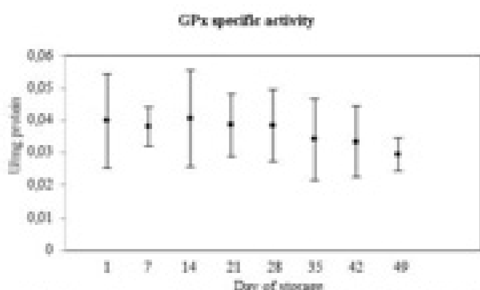

Fig. 6. The evolution of GPx enzymatic activity at RBCs banked for 49 days in CPDA. The values are expressed as U of enzyme/mg of protein and are related to an average concentration of 7.193.333 RBCs/µl. Tests were made in triplicate for RBC hemolyzate from each of the three donators. The error variations may be due to the specificity of donators.

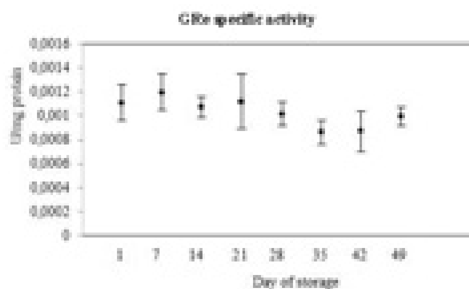

Fig. 7. The evolution of GRe enzymatic activity at RBCs banked for 49 days in CPDA. The values are expressed as U of enzyme/mg of protein and are related to an average concentration of 7.193.333 RBCs/µl. Tests were made in triplicate for RBC hemolyzate from each of the three donators. The error variations may be due to the specificity of donators (age, sex, lifestyle).

The decrease of SOD, CAT, GPx and GRe levels could indicate the alteration of antioxidant defense system of banked erythrocytes (Lynch *et al.*, 1977; Gaetani *et al*., 1989; Johnson *et al*., 1994; Scott *et al*., 1991). The decrease of thiol groups concentration could determine the decrease of antioxidant capacity and stability of plasmatic membrane at banked erythrocytes (Haest *et al*., 1983; Parker and Hoffman, 1964). We observed that the antioxidant enzymatic activities decreased after the first 7-14 days of storage that could indicate the moment when banked erythrocyte antioxidant capacity is overwhelmed by oxidant agents, leading to the amplifying of biological alteration. Thus, we could estimate that the equilibrium between antioxidant defense and alteration agents could break after 14 days of storage, irretrievably, with great negative consequences on cellular functions and morphology; this could result in aging and

even lisys of RBCs (Beutler, 1969; Zipursky *et al.*, 1973; Marjani *et al.*, 2007), like the fact we observed by confocal microscopy (fig. 12B-C).

Our results are similar to those of Dumaswala *et al.,* (1999), who reported a decrease of approx. 17% of erythrocyte CAT level, after 42 days of storage; according to them, after 42 days of storage erythrocyte GPx level decreased by approx. 12%. Our results are also similar to those of Szczypka *et al.* (1999), who reported a decrease of approx. 10% of banked erythrocyte SOD; according to them, GRe decreased by approx. 8% during 25 days of storage. However, they observed that GPx and CAT levels were almost constant. Marjani *et al.*, (2007) also observed that GPx and SOD levels decrease during blood banking, and Korgun *et al.*, (2001) observed an increase of lipid peroxidation in correlation with the decrease of antioxidant enzymatic concentration, at banked erythrocytes. The differences between our results and other could be due to the specificity of donators and to other factors that can influence metabolic activity of erythrocyte before blood collection (Marjani *et al.*, 2007).

Energy metabolism

Lactate dehydrogenase is an important player in the anaerobic generation of energy from glucose as well as in the synthesis of glucose from lactate. With lactate, LDH can produce NADH from NAD^+. Thus, LDH catalyzes the interconversion between pyruvate and lactate, concomitant with the interconversion between NADH and NAD^+. This enzyme converts pyruvate, the end product of glycolysis, into lactate in the absence of oxygen. At higher concentration of lactate, LDH generates an inhibition feedback which leads to decrease of pyruvate conversion. Also, sodium pyruvate exhibits protective activity against hydrogen peroxide (Giandomenico *et al.*, 1997). Thus, decrease of pyruvate concentration of banked erythrocytes could, also, indicate the decrease of erythrocyte antioxidant potential.

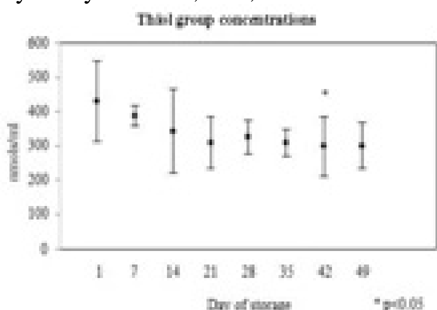

Fig. 8. The evolution of SH group concentration at RBCs banked for 49 days in CPDA. The values are expressed as nanomols/ml and are related to an average concentration of 7.193.333 RBCs/µl. Tests were made in triplicate for RBC hemolyzate from each of the three donators. Statistical analysis was made with Student's t-test. The error variations may be due to the specificity of donators (age, sex, lifestyle etc.).

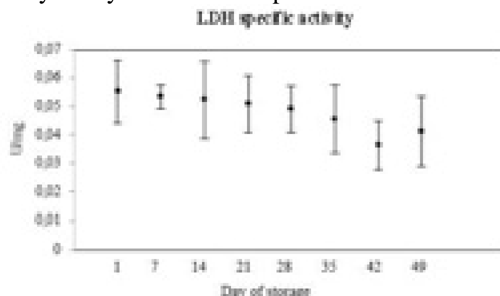

Fig. 9. The evolution of LDH enzymatic activity at RBCs banked for 49 days in CPDA. The values are expressed as U of enzyme/mg of protein and are related to an average concentration of 7.193.333 RBCs/µl. Tests were made in triplicate for RBC hemolyzate from each of the three donators.

In our study we observed that specific activity of LDH recorded a constant decrease of approx. 25% during storage period (fig. 9). Thus, we can conclude that generation of NAD^+ and NADH decreased as a result of LDH enzymatic activity in correlation with pyruvate concentration, which decreased too. Moreover, we can suppose that the entire erythrocyte energy generation by glycolysis decreased during storage, because of RBCs aging. This affirmation was sustained by pyruvate decreasing, which is the final product of glycolysis. Thus, we observed that pyruvate concentration exhibited a decreasing trend; from day of storage 7 until day 49 it decreased by approx. 26%, after an increase of approx. 16% between day 1 and day 7 (fig. 10). This, in correlation with the decrease of LDH level, can indicate the alteration of energy

metabolism potential/activity of erythrocytes as a result of their degradation/aging. Interesting is that the pyruvate level begun to decrease when the equilibrium between antioxidant defense-oxidative stress was broken. Thus, we can suppose that erythrocyte energy metabolism can be negatively influenced by oxidative stress during banking.

Cholesterol

Cholesterol is believed to be a moderating component in biomembranes (Bamberger *et al*, 1983; Hui *et al*, 1980). This kind of mobility exercised by cholesterol molecules also engages membrane lipids (Golan *et al*., 1984). In our study we observed that erythrocyte total cholesterol level decreased by approx. 34% during banking. The sharpest decline, of approx. 13%, was observed between day of storage 7 and day 14 (11). Between day 1 and day 7 the cholesterol level was approximately constant, same for the period between days 35 and 49. Thus, decrease of cholesterol concentration observed by us could influence the molecular arrangement of erythrocyte membrane lipids (Thompson *et al*., 1979). Therefore, the oscillations of cholesterol/phospholipid ration from plasmatic membrane influence its fluidity, which is important for the normal function of cell. Also, our observations could indicate that banked erythrocytes can suffer time-dependent loss in cholesterol correlated with the oxidative stress and aging status. Moreover, Studies of ghost membranes by fluorescent depolarization (Cooper *et al*., 1974) and photobleaching techniques (Thompson *et al*., 1979) have shown that the fluidity and the lateral mobility of lipids decrease more rapidly with temperature in cholesterol-depleted ghosts; this could represent a possible cause for banked erythrocyte deformation and fragmentation that we have observed by confocal microscopy. Our assumption is in correlation with significant decline of cholesterol concentration in the first 14 days of storage which was followed by morphological changes mentioned above. Thus, the loss of membrane cholesterol represents another element that alters lipid bilayer, because this can affect the mechanic stability of membrane causing morphological changes (Lange *et al*., 1981). Such changes include: ionic permeability modification (Cooper *et al*., 1975; Wiley *et al*., 1975), glycerol permeability (Bruckdorfer *et al*., 1969; Grunze *et al*., 1974), increase of membrane fragility (Bruckdorfer *et al*., 1969) and lateral mobility modification (McLean *et al*., 1981; Clejan *et al*., 1984).

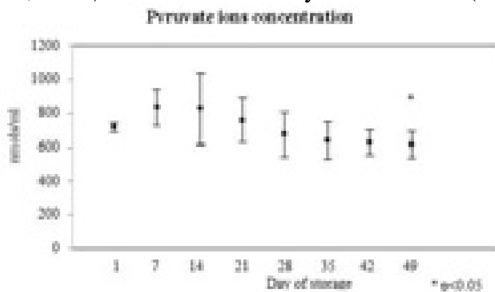

Fig. 10. Evolution of pyruvate ions concentration during RBC banki
The values are expressed as nanomoles/ml of hemolyzate and are rela
to an average concentration of 7.193.333 RBCs/µl. Tests were made
triplicate for RBC hemolyzate from each of the three donators.

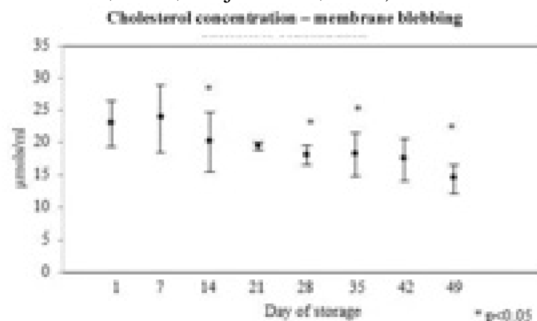

Fig. 11. Cholesterol concentrations recorded during RBC banking. Values expressed as micromoles/ml of hemolyzate and are and are related to average concentration of 7.193.333 RBCs/µl. Tests were made in triplicate RBC hemolyzate from each of the three donators. Statistical analysis made with Student's t-test.

Fluorescent marking of erythrocyte membranes – hypericin

For highlighting erythrocyte membrane changes which occur during storage we used hypericin, as a new erythrocyte membrane fluorescent marker. Thus, we were able to observe when plasmatic membrane of banked erythrocytes begun to deform and to fragment. This

observation was further correlated with the biochemical data regarding oxidative stress and erythrocyte alteration during banking. Because of its lipophilic character, hypericin binds preferentially the lipid matrix of cell membrane, especially the cholesterol rich zones, which represent the main target (Ho *et al.*, 2009; Jonas *et al.*, 2008; Phillips *et al.*, 1992). Hypericin is a natural compound, easy to obtain and not toxic. Moreover, it poses high fluorescent efficiency.

After collection we observed that erythrocytes membrane didn't suffer significant changes (fig. 12A). However, there was reported a small number of erythrocytes that modified lightly their shape and had a small hypericin intake, probably due to the *in vivo* physiological status of collected erythrocytes (not all collected RBCs are at the same age at the same time). Further, we observed that more erythrocytes begun to exhibit a decrease of membrane fluorescence; this could indicate that RBCs had a time dependent membrane cholesterol loss, what was confirmed by our biochemical results. Besides fluorescent changes, many banked erythrocytes suffered time dependent deformation and hypericin intake, emphasizing the membrane destabilization (fig. 12B). We observed that over time low fluorescence of aged erythrocyte membranes changed into diffuse fluorescence, membranes were more deformed or even fragmented, suggesting the increasing alteration of RBC membrane integrity and functionality; also, the hypericin intake has increased progressively, in a time dependent manner (fig. 12C). The hypericin intake at aged erythrocytes could be explained by the increased permeabilization of RBC membranes caused by the oxidative stress and cholesterol loss. Thus, hypericin penetrated the membrane, into the cell, where bound the cytosolic cholesterol (Ho *et al.*, 2009). Alteration or loss of membrane cholesterol can cause important morphological changes of lipid molecules arrangement (Thompson *et al.*, 1979); as a result, excessive permeabilization may occur. Another reason for the hypericin intake could be membrane fragmentation of RBCs, due to its degradation. Although, the low and/or diffused fluorescence observed at membrane regions and, also, hypericin intake may indicate the progressive state of erythrocyte degradation/age.

Hypericin binding to erythrocyte membranes

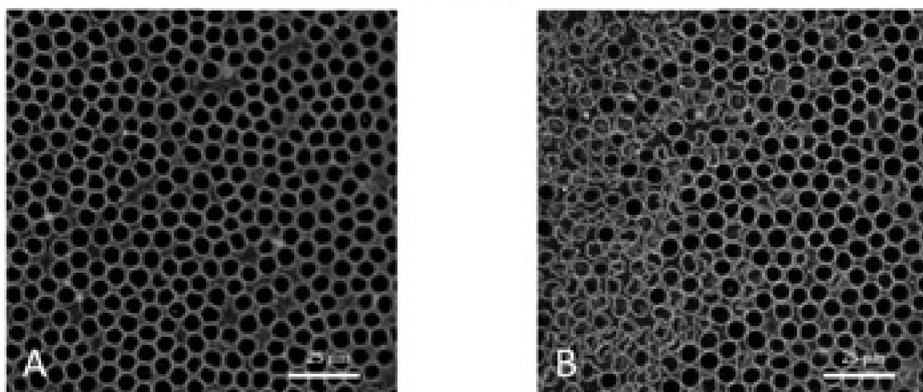

Fig. 12. A, erythrocytes are in the first day of banking. Hypericin was attached to the RBC membrane highlighting its shape and integrity; erythrocytes showed no deformation or any other signs of deterioration. Only few RBC exhibited low deformation because not all cells are at the same age at collection. In figure 12 B are presented RBCs in day of storage 21. We observed that many erythrocytes suffered strong deformation caused by membrane alteration, in result of oxidative damage (when protein oxidation reached its maximum and antioxidant defense decreased). Also, RBCs exhibited hypericin intake, sign of strong membrane alteration. Some erythrocytes showed lower membrane fluorescence than in the first day of storage, probably as result of cholesterol loss. There was also observed fluorescent intercellular zones that supposed to be cytosolic content, probably indicating RBC lisys.

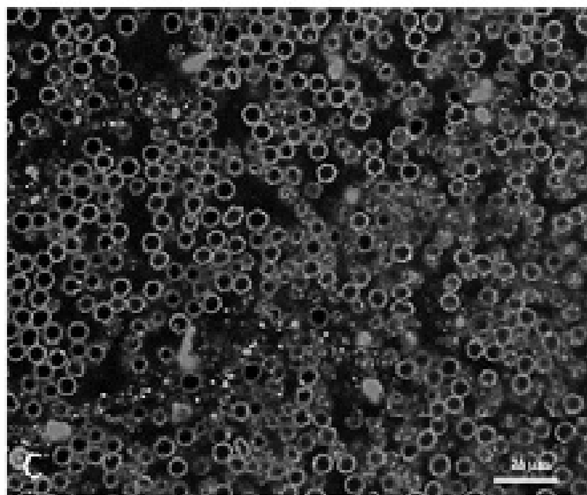

Fig. 12 C. Erythrocytes are in day of storage 42. We observed that after 6 weeks banked RBCs showed very strong membrane alteration, including significant deformation, excessive permeabilization, and fragmentation (demonstrated by increasing amount of hypericin intake). As a result, few erythrocytes remained in good shape for a possible transfusion. Also, we observed that the intracellular fluorescence significantly increased in intensity, possibly indicating a very high level of RBC lysis caused by oxidative damage. More RBCs exhibited low membrane fluorescence, indicating membrane degradation. All these morphological changes may suggest that RBCs suffer a rapid aging caused by oxidative stress during storage in CPDA. Thus, we showed that hypericin can represent a sensitive RBC membrane marker, highlighting in detail morphological alteration of banked erythrocytes, and quality of blood respectively.

CONCLUSIONS

In this study we observed that erythrocytes banked in CPDA solution suffered increased oxidative stress, lipid peroxidation and protein oxidation respectively, during banking. The erythrocyte antioxidant defense potential decreased in correlation with the increase of oxidative stress. The oxidant agents altered the membrane cholesterol decreasing its concentration that led to the destabilization of lipid bilayer and to the excessive permeabilization of RBC membranes. All these factors degraded the plasma membrane leading to increased membrane permeabilization, rigidity, deformation and/or fragmentation and to an increased level of hemolysis. When the equilibrium between antioxidant defense and oxidant agents was broken the energy potential of erythrocytes also decreased.

Our fluorescence method of erythrocyte marking with hypericin managed to highlight, in a great detail, the progressive degradation of erythrocyte morphology. We observed that old erythrocytes exhibited hypericin intake that was grater with the increasing of age. Also, aged erythrocytes presented a lower fluorescence against the older ones. The advanced degradation state of erythrocyte membrane was highlighted by diffused fluorescence. During banking, the number of reliable RBCs progressively decreased. We observed, by hypericin fluorescence, how erythrocyte membrane deformed or fragmented during storage period. By marking with hypericin we also observed the vesiculation phenomena and that the cytosolic content is excluded out of cells due to the excessive permeabilization and/or to the fragmentation of membrane. Thus, according to the amplitude of these membrane lesions we can appreciate the quality of banked blood. Through this biochemical and imaging studies we demonstrated that hypericin could

represent a new erythrocyte membrane marker capable of fluorescence changes correlated to the morphologic alteration caused by degradation of erythrocytes.

Because of our results we suggest that storage of erythrocytes, for more than 21 days, causes a significant decrease of blood quality. Nevertheless, further studies must be made on a larger number of donators with a similar physiological and medical profile (same age, sex, lifestyle etc.). The utility perspective of out fluorescence method of erythrocyte membrane marking could be extended for highlighting morphological changes caused by oxidative stress to the erythrocytes from patients suffering different types of anemia, diabetes and hypertension.

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