Original Paper

Natural Antioxidants Improve Red Blood Cell “Survival” in Non-Leukoreduced Blood Samples

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Key Words

Red blood cells • Alsever's solution • Carnosine • Spermine • Phloretin • Phosphatidylserine exposure • Calcium

Abstract

Background: Blood collected in an anticoagulant can be kept refrigerated in an unmodified state within 5 - 6 weeks. Oxidative damage is considered to be a one of the major factors contributing to the development of storage lesions. Lipid and membrane proteins oxidation results in changes in cation gradients that affect the cell survival. **Aim:** In the present study we used the natural antioxidants and ion channels blockers (L-carnosine, spermine, phloretin and their mixtures) to prolong “survival” of red blood cells (RBCs), measured as the lack of PS exposure and cell hemolysis, in the Alsever's preservative solution upon hypothermic storage. **Results:** We show that the mixture of carnosine (20 mM), spermine (20 μM) and phloretin (100 μM) effectively blunted phosphatidylserine (PS) exposure, Ca²⁺ accumulation and RBCs hemolysis in non-leukoreduced low (~2%) hematocrit samples after 36 days of storage as well as after 1 day of post-storage incubation of the stored cells in physiological saline solution. In addition, a slight but significant decrease in PS exposure was observed in non-leukoreduced high (~20%) hematocrit samples after 36 days of storage with the mixture of substances. **Conclusion:** We conclude that the use of the mixture of natural antioxidants (carnosine, spermine, and phloretin) as an additive to blood preservative solution provides better RBCs storage and “survival”.

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Introduction

Effective preservation and long-term storage of human red blood cells (RBCs) followed by transfusion of the cells to recipient is one of the most important problems in medicine.

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According to the World Health Organization (WHO) about 107 million blood donations were collected worldwide in 2013. Beside the problems connected with sterility of the blood samples (the absence of pathogenic infection and viruses) there is a general problem of cell survival during the storage and after the transfusion. Cryopreservation of RBCs in highly concentrated glycerol media results in about 80% survival of de-frozen cells [1, 2]. However, the method complexities (expensive materials used and post-storage cryoprotectant removal problems) limit the use of cryopreserved blood. Hypothermic storage becomes more popular since no cryoprotectants are used, the cells could be kept in standard refrigerators at +4 °C up to 7-10 weeks and could be transfused immediately upon demand. The composition of the media for hypothermic storage involves Ca^{2+} -binding compounds (EDTA/EGTA, citrate), reducing sugars (glucose, mannitol, and sorbitol) and inorganic phosphates to maintain the level of ATP and 2, 3-DPG in the cells.

However, the use of hypothermia for cell storage is limited due to biological and biochemical alterations, known as storage lesions, that include changes in metabolism and cation gradients, oxidation, vesiculation, loss and cross-linking of band 3, carbonyl modification of band 4.1 [3, 4], elevated PS exposure, reduced deformability and irreversible echinocytosis, hemolysis [5-7]. According to the current theory of storage lesion, the fall in ATP level weakens the cell antioxidant defence resulting in a decline in glutathione peroxidase activity [3, 8] and cell damage by reactive oxygen species (ROS). The levels malondialdehyde (MDA), ascorbate (ASC), dehydroascorbic acid (DHA), interleukin-6 (IL-6) were shown to increase significantly during the storage period [9, 10] suggesting a progressive oxidative stress in preserved cells. It causes disruption of the cytoskeleton, aggregation of band 3 and release of vesicles [5-7, 11, 12]. At the same time, lipid peroxidation facilitates formation of advanced glycation end products (AGEs). The levels of HbA_{1c} , a marker of RBC ageing [13] as well as AGE-modified cell surface membrane proteins were reported to increase over storage progression [14-16]. Storage of RBCs in anaerobic conditions was shown to reduce significantly oxidative damage, the rate of hemolysis and PS exposure and, thus, prolonged the cells viability [7].

It is known that about 25% of long-stored (more than 5 weeks) RBCs are immediately removed from the circulation during the post-transfusion [17]. The recent data showed that overnight incubation at +37 °C of 35 days stored RBCs increased cation leakage, hemolysis, PS exposure and vesiculation [18]. Increased cation leakage in stored RBCs could be due to enhanced cation permeability of peroxidised lipid bilayer as well as to activation of the non-selective Ca^{2+} - permeable cation channels. According to patch-clamp data, that give the evidence of functioning ion channels, there are two types of non-selective Ca^{2+} - permeable cation channels in erythrocytes: (i) voltage-gated channel, coupled to an acetylcholine receptor [19-21], and (ii) partially related to TRPC6 channels [22] voltage-independent cation channel [23]. The latter was shown to be stimulated by osmotic shock and oxidative stress, the events which accompany cell ageing [24-26]. Recent data demonstrated also the existence of NMDA receptors in rat and human [27-29] and AMPA receptors in human [30] erythrocytes. In addition, the presence of subtypes of voltage-dependent Ca^{2+} channels was demonstrated by Western blot analysis in age-fractionated erythrocytes [31].

The role of Ca^{2+} - permeable non-selective cation channels in stimulation of suicidal cell death (also known as eryptosis) is well documented. Eryptosis is known to be triggered by an increase of cytosolic Ca^{2+} concentration that results in enhanced cell membrane scrambling, cell membrane blebbing, and cell shrinkage [32-35]. Transfusion of hypothermically stored cells, kept in low Ca^{2+} -containing preservative media, induces activation of Ca^{2+} conductance and thus can stimulate suicidal cell death. Eryptotic cells are quickly eliminated from the blood stream by lymphocytes and macrophages increasing the liver and spleen overload.

Our recent data [36-38] revealed new mechanisms for the non-selective (Ca^{2+} -permeable) cation channels regulation in RBCs. The natural cell component polyamine spermine at micromolar concentrations blocks channels activated by Cl^- removal as well as the basal activity of the channels in physiological Cl^- -containing saline [37]. Moreover, spermine was shown to modulate activity of AMPA/kainate and NMDA receptors in neurons and glial cells

[39-40]. Taking into account an anti-oxidative effect of micromolar spermine concentrations [37] we assumed that spermine could be an effective component of preservation solution for hypothermic storage of RBCs.

Natural ageing as well as eryptosis result in osmotic cell water loss and cell shrinkage. Recently we showed that aquaporin 9 (AQP9) contributes to cation permeability of erythrocytes either acting as a cation-conducting channel or by regulating the activity of other cation channels [38]. Addition of phloretin, a natural antioxidant [41-42] that blocks the glucose transporter [43] and water channels [44], to RBC preservative solution could prevent the cell shrinkage and, thus, increase the cell survival.

Non-enzymatic glycation is a non-enzymatic irreversible process that is promoted by the prolonged exposure of RBCs to high glucose concentrations, a condition that is known to occur under low temperature blood preservation [15]. Progressive accumulation of glycated end products was observed upon hypothermic storage of RBCs [14]. Our recent data [36] demonstrated that glycated human RBCs showed enhanced activity of the non-selective (Ca^{2+} -permeable) cation channels that results in $[\text{Ca}^{2+}]_i$ increase, cell shrinkage and PS exposure. We supposed that L-carnosine, ROS scavenger [45] and inhibitor of non-enzymatic glycation [46], when used as a component of blood preservative solution, could prevent formation of glycated end products and improve stored cell survival.

Thus, in the present study we used the known antioxidants (L-carnosine, spermine and phloretin) to prolong RBCs "survival", measured as the lack of PS exposure and cell hemolysis (if detected), in Alsever's protective solution upon 36 days of hypothermic storage.

Materials and Methods

Blood

Experiments with human RBCs were authorized by the ethics committee of the medical association of the Saarland under registration number 132/08. Blood donors provided their written informed consent to participate in this study. For the experiments, we used RBCs from healthy adult donors. Blood was vein drawn into heparinized syringes.

Sample preparation

The blood samples for long-term storage at +4 °C were made under sterile conditions. Sterile Alsever's preservative solution (Sigma, Germany) was used.

Low hematocrit (approximately 2%) and high hematocrit (approximately 20%) samples were made by mixing whole blood with Alsever's solution in the presence of 10 and 20 mM carnosine; 20 and 200 μM spermine; 100 μM phloretin; and the mixtures of 20 mM carnosine and 20 μM spermine; 20 mM carnosine 20 μM spermine and 100 μM phloretin. The blood samples preserved in Alsever's solution without additives were used as controls. Alsever's solution contained (g/L): 4.2 NaCl, 8 Na-citrate * 2 H_2O , 0.55 citric acid and 20.5 D-glucose.

The samples stored at +4 °C were checked for cytosolic free Ca^{2+} concentration (Fluo4 fluorescence) and PS exposure (Annexin V binding) at 1, 3, 8, 22 and 36 days of storage.

After 36 days of hypothermic storage the preservative media were removed (short spin, 15 s, 16,000 g), the cells were re-suspended in physiological saline solution (pH 7.4) and incubated for 24 h at +37 °C. Physiological saline solution consisted of (in mM): 145 NaCl, 7.5 KCl, 2 CaCl_2 , 10 glucose and 10 HEPES (pH 7.4 with NaOH). Then the cells were again checked for PS exposure (Annexin V binding).

Hemolysis in the supernatants was measured after the hypothermic storage (36 days) and after 1 day incubation in physiological saline solution.

Intracellular free Ca^{2+} measurements

Intracellular free Ca^{2+} was analyzed by flow cytometry using the Ca^{2+} -sensitive probe Fluo4. RBCs (0.1% hematocrit) were loaded with Fluo4 (1 μM) and incubated for 30 min at +37 °C. Then extracellular Fluo4 was removed (10 s, 16,000 g, Eppendorf, 5415 D centrifuge) by aspiration and the cell pellet was re-suspended in physiological saline solution. The cells (30,000) were then analyzed using a flow cytometer (FACS-Calibur, Becton Dickinson Biosciences, USA) in FL-1 channel with 488 nm excitation and 530 nm

emission wavelengths. The data were processed using BD Cell Quest Pro Software (Becton Dickinson Biosciences, USA).

Phosphatidylserine exposure measurements

The cells (0.1% hematocrit) were stained with Annexin V-FITC (Invitrogen, Molecular Probes, Germany) at a 1:125 dilution in 2 mM Ca^{2+} -containing physiological saline solution. After 20 min of incubation samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; USA). Annexin V-FITC fluorescence intensity was measured in fluorescence channel FL-2 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Hemolysis measurements

RBC hemolysis was estimated as oxyhemoglobin (Oxy-Hb) release. Oxy-Hb was measured in supernatants with UV-VIS spectrophotometer (UVmini-1240, Shimadzu, Japan) at 577 nm wavelength. Concentration of Oxy-Hb was calculated from the adsorption data using the molar extinction coefficient of $14.6 \text{ mM}^{-1} \times \text{cm}^{-1}$ per heme.

Chemicals and solutions

All the chemicals used were of the highest grade. Alsever's solution, carnosine and phloretin were from Sigma (Germany), spermine from Roth (Germany).

Statistics

The experiments were done in triplicate using blood of different blood donors. Data are presented as arithmetic means $\pm$ SEM. Unpaired two-tailed t-test was employed as appropriate, $p < 0.05$ and $p < 0.01$ were considered statistically significant.

Results

Alsever's solution, an isotonic, anticoagulant blood preservative, had proved satisfactory with the usual criteria for studying RBC preservation; that is, rate of spontaneous hemolysis, fragility tests, hemoglobin levels, and incidence of reactions. However, ageing of RBCs preserved in Alsever's solution was not tested. In the present study we explored the changes in the cells "survival", measured as the lack of PS exposure and (in some cases) hemolysis, during 36 days storage of blood in Alsever's solution.

In the first series of experiments we used high (~20%) hematocrit blood samples prepared according to the standard procedure by mixing one part of Alsever's solution with one part of whole blood.

Fig. 1A shows changes in Annexin V binding of the RBCs upon storage in Alsever's solution. No significant changes in Annexin V binding were observed in the cells during the first 2 weeks of storage. Percentage of PS-exposing cells significantly increased after 3 weeks of storage and reached the value of 6.93 ± 0.36 after 5 weeks of hypothermic storage.

The use of carnosine (10 and 20 mM, Fig. 1B and C) and spermine (20 μM and 200 μM , Fig. 1D and E) as additives to Alsever's solution did not significantly improve the cell "survival". In opposite, 100 μM phloretin alone (Fig. 1F) and in combination with carnosine and spermine (Fig. 1G and H) blunted PS exposure after 6 weeks of cell storage. PS exposure in the cells suspended in Alsever's solution containing carnosine, spermine and phloretin was significantly lower than in control cells (4.41 ± 0.03 versus 6.93 ± 0.36 for control cells) after 36 days of storage (Fig. 2A, B and E).

Since PS exposure could be due to an increase in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), with stimulation of Ca^{2+} -dependent scramblase activity, we measured the changes in $[\text{Ca}^{2+}]_i$ during cell storage. As it is shown in Fig. 1A, a cell population with elevated Fluo4 intensity (highly Fluo4 fluorescent cells) tended to increase upon cell storage, indicating a total $[\text{Ca}^{2+}]_i$ increase. However, no linear correlation between $[\text{Ca}^{2+}]_i$ and PS exposure in the cells preserved in Alsever's solution either in the absence or presence of the additives was

Fig. 1. Time-dependent changes in Annexin V binding and Fluo4 fluorescence in high (~20%) hematocrit blood samples preserved with Alsever's solution. A. Annexin V binding (closed squares, $n = 3$) and highly Fluo4 fluorescent (open squares, $n = 3$) RBCs (% of cells), stored (+4 °C) for 1, 3, 8, 22 and 36 days in Alsever's preservative solution. B. as in A. in the presence of 10 mM carnosine. C. as in A. in the presence of 20 mM carnosine. D. as in A. in the presence of 20 μ M spermine. E. as in A. in the presence of 200 μ M spermine. F. as in A. in the presence of 100 μ M phloretin. G. as in A. in the presence of 20 mM carnosine and 200 μ M spermine. H. as in A. in the presence of 20 mM carnosine, 200 μ M spermine and 100 μ M phloretin.

found. Moreover, we observed a significant increase in $[Ca^{2+}]_i$ and a decrease in Annexin V binding in the cells suspended in the carnosine containing Alsever's solution (Fig. 1 and 2).

At the same time, carnosine blunted PS exposure as well as $[Ca^{2+}]_i$ increase in highly diluted (~2% hematocrit) blood samples (Fig. 3 and Fig. 4).

Similarly to what was observed with high hematocrit blood samples, the mixture consisting of carnosine, spermine and phloretin was the most effective in protection of low hematocrit blood samples against increase of PS exposure (4.84 ± 0.86 for the mixture treated cells in comparison with 15.72 ± 1.91 for control cells) after 36 days of storage (Fig. 3H and Fig. 4). Moreover, the cell samples preserved with the mixture accumulated significantly less Ca^{2+} than control cells (7.36 ± 0.03 versus 9.4 ± 0.23 for control cells).

Noteworthy, spermine alone at high concentration (200 μ M) demonstrated a strong protection, blocking the time-dependent increase in $[Ca^{2+}]_i$ and PS exposure in low hematocrit blood samples (Fig. 3E and Fig. 4). The effect was comparable with the effect of the carnosine-spermine-phloretin mixture (Fig. 4). The data on low hematocrit blood samples showed that, in most cases, PS exposure and intracellular free Ca^{2+} levels were coupled. Thus, enhanced PS exposure in the preserved cells was paralleled by an increase in $[Ca^{2+}]_i$.

Fig. 2. Annexin V binding and Fluo4 fluorescence in high (~20%) hematocrit blood samples stored at +4 °C in Alsever's solution for 36 days. A., B. Flow cytometric histograms of Annexin V binding in representative experiments on blood samples stored at +4 °C for 36 days in Alsever's solution in the absence (A.) and the presence of the mixture consisting of 20 mM carnosine, 200 μM spermine and 100 μM phloretin (B). C., D. Flow cytometric histograms of Fluo4 fluorescence in representative experiments on blood samples stored at +4 °C for 36 days in Alsever's solution in the absence (C.) and the presence of the mixture consisting of 20 mM carnosine, 200 μM spermine and 100 μM phloretin (D.). E. Arithmetic means ± SEM of Annexin V binding (%) in blood samples stored at +4 °C for 36 days in Alsever's solution in the absence of additives (white bar, n = 3), in the presence of 10 mM and 20 mM carnosine (light grey bars, n = 3), 20 μM and 200 μM spermine (grey bars, n = 3), 100 μM phloretin (dark grey bar, n = 3), mixture of 20 mM carnosine and 200 μM spermine (dark grey bar, n = 3) and mixture of 20 mM carnosine, 200 μM spermine and 100 μM phloretin (black bar, n = 3). * indicates significant difference from control cells (p<0.05; unpaired t-test). F. as in E. for Fluo4 fluorescence. * and ** indicate significant difference from control cells (p<0.05 and p<0.01, respectively; unpaired t-test).

In the next series of experiments we modeled the situation of transfusing the stored cells to a patient to check the cell "survival" in blood stream. For this, we incubated the 36 days stored cells in physiological saline solution for 1 day at 37 °C and then measured Annexin V binding to the cells and oxy-Hb release in the cell supernatants as an index of cell lysis.

As it is shown in Fig. 5 (compare with control samples of Fig. 2 and Fig. 4, Annexin V binding), 1 day treatment in physiological saline solution of control cells preserved in Alsever's solution dramatically stimulated Annexin V binding in high-hematocrit (6.93 ± 0.36 and 31.09 ± 2.91 , before and after the incubation, respectively) as well as in low-hematocrit (15.72 ± 1.91 and 35.66 ± 1.88 , before and after the incubation, respectively) blood samples.

The high-hematocrit cell samples preserved with the mixture of carnosine, spermine and phloretin did not show a significant difference in Annexin binding levels in comparison with control cells after the incubation in physiological saline solution (Fig. 5A).

In opposite, the incubation (1 day, +37 °C) of low-hematocrit blood samples, preserved with the mixture of carnosine, spermine and phloretin, resulted in a significantly lower value of PS exposing cells (9.32 ± 1.18 versus 35.66 ± 1.89) in comparison with control cells preserved in Alsever's solution without additives (Fig. 5B). Interestingly, all additives and mixtures, except 20 μM spermine (that was ineffective) and 100 μM phloretin (that highly enhanced PS exposure), significantly blunted PS exposure in low hematocrit blood samples upon the post-incubation (Fig. 5B).

Fig. 3. Time-dependent changes in Annexin V binding and Fluo4 fluorescence in low (~2%) hematocrit blood samples preserved with Alsever's solution. A. Annexin V binding (closed squares, $n = 3$) and highly Fluo4 fluorescent (open squares, $n = 3$) RBCs (% of cells), stored (+4 °C) for 1, 3, 8, 22 and 36 days in Alsever's preservative solution. B. as in A. in the presence of 10 mM carnosine. C. as in A. in the presence of 20 mM carnosine. D. as in A. in the presence of 20 μM spermine. E. as in A. in the presence of 200 μM spermine. F. as in A. in the presence of 100 μM phloretin. G. as in A. in the presence of 20 mM carnosine and 200 μM spermine. H. as in A. in the presence of 20 mM carnosine, 200 μM spermine and 100 μM phloretin.

In addition, the post-incubation of high hematocrit cell samples preserved with the mixture of carnosine, spermine and phloretin did not enhance significantly the cell hemolysis (measured as free oxyhemoglobin release in supernatants). However, in low hematocrit cell samples the mixture of carnosine, spermine and phloretin effectively protected the cells against destruction (Fig. 6).

Discussion

The present study was performed with Alsever's blood preservative solution. The solution was developed in the USA during the World War II and it is still routinely used as whole blood (or packed RBCs) preservative and is the only blood preservative available from Sigma. Maximal storage time for blood preserved with Alsever's solution is announced as 10 weeks. In our study, however, a shorter storage period (36 days) that is typically used in blood preservation practice was applied.

Literature data showed that antioxidants given either to blood donors [47] or to storage solutions [2, 48] improve RBCs storage parameters by reducing free radical cell damage. The most promising results could be expected when antioxidants effectively inhibit cation

Fig. 4. Annexin V binding and Fluo4 fluorescence in low (~2%) hematocrit blood samples stored at +4 °C in Alsever's solution for 36 days. A., B. Flow cytometric histograms of Annexin V binding in representative experiments on blood samples stored at +4 °C for 36 days in Alsever's solution in the absence (A.) and the presence of the mixture consisting of 20 mM carnosine, 200 μM spermine and 100 μM phloretin (B). C., D. Flow cytometric histograms of Fluo4 fluorescence in representative experiments on blood samples stored at +4 °C for 36 days in Alsever's solution in the absence (C) and the presence of the mixture consisting of 20 mM carnosine, 200 μM spermine and 100 μM phloretin (D). E. Arithmetic means ± SEM of Annexin V binding (%) in blood samples stored at +4 °C for 36 days in Alsever's solution in the absence of additives (white bar, n = 3), in the presence of 10 mM and 20 mM carnosine (light grey bars, n = 3), 20 μM and 200 μM spermine (grey bars, n = 3), 100 μM phloretin (dark grey bar, n = 3), mixture of 20 mM carnosine and 200 μM spermine (dark grey bar, n = 3) and mixture of 20 mM carnosine, 200 μM spermine and 100 μM phloretin (black bar, n = 3). * indicates significant difference from control cells (p<0.05; unpaired t-test). F. as in E. for Fluo4 fluorescence. * and ** indicate significant difference from control cells (p<0.05 and p<0.01, respectively; unpaired t-test).

leakage [49, 50]. Based on our recent data [36 - 38] we selected three substances (carnosine, spermine and phloretin) that meet the above criteria, i.e. they are antioxidants of natural origin that show blocking effects on cation channels.

We supposed that the use of carnosine, spermine and phloretin as additives to blood preservative solutions could provide better "survival" of RBCs during storage and after the cells transfusion.

The experiments were performed with high and low hematocrit non-leukoreduced blood samples. It allowed us to study the effect of blood components on the stored RBC "survival". Since the life-time of white blood cells and platelets is short in comparison with RBCs, varying from hours to 3-4 days for white cells and 5-9 days for platelets, plasma components and the substances released by dead leukocytes/platelets can affect preserved RBCs survival. Residual white blood cells and platelets were shown [51] to represent a stressful storage factor, countering the structural and functional integrity of stored RBCs. Hemolysis, irreversible echinocytosis, microvesiculation, ROS and Ca²⁺ accumulation, band 3 related senescence modifications, membrane proteome stress biomarkers were recorded mostly in non-leukoreduced RBCs compared to leukoreduced RBCs. Plasma could have a dual

Fig. 5. Annexin V binding in the hypothermically preserved cells after 24 h incubation at +37 °C in physiological saline solution. A. Annexin V binding in high (~20%) hematocrit samples preserved in Alsever's solution (+4 °C, 36 days) solution in the absence of additives (white bar, n = 3), in the presence of 10 mM and 20 mM carnosine (light grey bars, n = 3), 20 μ M and 200 μ M spermine (grey bars, n = 3), 100 μ M phloretin (dark grey bar, n = 3), mixture of 20 mM carnosine and 200 μ M spermine (dark grey bar, n = 3) and mixture of 20 mM carnosine, 200 μ M spermine and 100 μ M phloretin (black bar, n = 3), then incubated for 24 h at +37 °C in physiological saline solution. B. as in A. for low (~2%) hematocrit cells.

Fig. 6. Free oxy-Hb release in the hypothermically preserved cells after 24 h incubation at +37 °C in physiological saline. A. Hemolysis, measured as free oxy-Hb release in supernatants of high (~20%) hematocrit samples preserved in Alsever's solution (+4 °C, 36 days) solution in the absence of additives (white bar, n = 3), in the presence of 10 mM and 20 mM carnosine (light grey bars, n = 3), 20 μ M and 200 μ M spermine (grey bars, n = 3), 100 μ M phloretin (dark grey bar, n = 3), mixture of 20 mM carnosine and 200 μ M spermine (dark grey bar, n = 3) and mixture of 20 mM carnosine, 200 μ M spermine and 100 μ M phloretin (black bar, n = 3), then incubated for 24 h at +37 °C in physiological saline solution. B. as in A. for low (~2%) hematocrit cells.

effect on RBCs survival. It is known to possess an antioxidant activity and, thus, to protect the cells against oxidative stress. The major substances contributing to total antioxidant capacity of plasma are free radical scavengers (albumin, urate, ascorbate, bilirubin), metal-binding antioxidants (caeruloplasmin and transferrin) and vitamins (alpha tocopherol, beta-carotene, retinol and ascorbic acid, cobalamin) [52, 53]. However, plasma components could also interact with additives present in blood preservative solutions, resulting in the formation of cytotoxic products [54]. Moreover, plasma proteins (serum albumin, apolipoprotein A1, transferrin) undergo oxidation [55] and non-enzymatic glycation [56] upon storage in sugar enriched protective media that result in increased oxidative damage of stored RBCs.

Table 1. RBCs "survival" (measured as % of cells exposing no PS) and oxy-Hb release (mM) in the cells suspensions (~2 and ~20 % hematocrit) preserved (+4 °C, 36 days) in Alsever's solution in the absence (control) or presence of the mixture of 20 mM carnosine, 20 μ M spermine and 100 μ M phloretin and then incubated (24 h, +37 °C) in physiological saline solution. * and ** indicate significant difference from control ($p < 0.05$ and $p < 0.01$, respectively; unpaired t-test). # No oxy-Hb release was detected in the cells samples after 36 days storage at +4 °C

		RBCs "survival", % cells		oxy-Hb release, mM	
		storage	incubation	storage#	incubation
~2% hematocrit	control	87.38 $\pm$ 1.41	64.34 $\pm$ 1.89	-	0.46 $\pm$ 0.08
	mixture	95.16 $\pm$ 0.86*	90.68 $\pm$ 1.18**	-	0.18 $\pm$ 0.01
~20% hematocrit	control	93.07 $\pm$ 0.36	69.48 $\pm$ 1.35	-	0.62 $\pm$ 0.02
	mixture	95.59 $\pm$ 0.27*	63.37 $\pm$ 2.84	-	0.61 $\pm$ 0.01

The effectiveness of the additives to Alsever's protective solution, used in our study, was estimated as RBC "survival" after 36 days of storage and after post- incubation. In our experiments we modelled transfusion by incubation of the stored cells in physiological Ca^{2+} -containing saline for 24 h at +37 °C. Opposite to the commonly used radioisotope method of a 24 h RBC survival study, where the stored cells are tagged with chromium isotope (^{51}Cr), re-infused, and the percentage of cells which survive in the circulation at 24 h is measured, we estimated RBCs "survival" as percentage of Annexin V binding and oxy-Hb release due to RBC hemolysis.

Our data demonstrated that PS exposure of either high or low hematocrit blood samples show an exponential increase upon storage. High hematocrit RBCs demonstrated slower increase in PS exposure in comparison with low hematocrit blood samples. The changes in PS exposure may reflect storage related changes in intracellular ATP concentration and endogenous cell antioxidant system. It was shown that RBCs stored under blood bank conditions are capable of attenuating ROS [10] and maintaining unchanged intracellular ATP levels within 2 weeks of storage [57]. Oxidative stress, ATP deprivation and cytosolic Ca^{2+} depletion induce deactivation of PMCA, facilitate Ca^{2+} transport and evoke acute Ca^{2+} overload in the transfused cells [58]. Elevated cytosolic Ca^{2+} concentration, in turn, can trigger enhanced cell membrane scrambling.

Our data showed that high hematocrit RBCs stored for 36 days in Alsever's solution without additives "survived" better than low hematocrit RBCs (93.07 $\pm$ 0.36 % *versus* 87.38 $\pm$ 1.41 %; Table 1). The effect could be due to antioxidant plasma activity.

Only the 3-components mixture of carnosine, spermine and phloretin slightly but significantly enhanced RBC "survival" in high hematocrit blood samples (95.59 $\pm$ 0.27 % *versus* 93.07 $\pm$ 0.36 %; Table 1). Noteworthy, a decrease in PS exposure was accompanied with a significant increase in $[\text{Ca}^{2+}]_i$. Carnosine (alone or as a mixture component) enhanced $[\text{Ca}^{2+}]_i$ in high hematocrit RBCs. The result seems to be surprising since carnosine is known to protect the cells against non-enzymatic glycation [46, 59, 60]. Our recent data showed that glycation activated in isolated RBCs the non-selective Ca^{2+} -permeable cation channel sensitive to inhibition with 2 mM amiloride [36]. Glycation inhibitors might protect the cells against Ca^{2+} overload due to the channel activation and, thus, increase the cells survival via inactivation of the Ca^{2+} -dependent scramblase. However, the known effects of carnosine are diverse and they do not include only ROS scavenging and non-enzymatic glycation inhibition. Carnosine was shown to increase $[\text{Ca}^{2+}]_i$ in the isolated rat heart via stimulation of Ca^{2+} release from the ryanodine calcium-release channel, inhibition of Ca^{2+} uptake by the sarcoplasmic reticulum Ca^{2+} pump, and sensitization of the contractile proteins to Ca^{2+} [61]. Moreover, non-leukoreduced high hematocrit blood samples are complex systems. The dead buffy coat cells and plasma, presented in high concentrations in the samples, could interact with the components of Alsever's solution changing their properties. As an example,

low protective efficiency of 200 μM spermine in high hematocrit blood samples could be due to interaction of spermine with plasma. It is known that spermine can be oxidized by plasma and cellular aminooxidases resulting in the formation of cytotoxic products, such as aminoaldehydes, NH_3 , and H_2O_2 [54].

In opposite to high hematocrit blood samples, carnosine demonstrated some protective effect in low hematocrit blood samples (Fig. 4). Added together with spermine and phloretin, carnosine significantly blunted intracellular free Ca^{2+} accumulation and improved RBC "survival" ($95.16 \pm 0.86\%$ versus $87.38 \pm 1.41\%$, Table 1) after 36 days of storage. Moreover, in agreement with our previous results on isolated RBCs [37] that showed that spermine, in the high micromolar range, blocked the non-selective Ca^{2+} permeable channels and PS exposure, our data demonstrate higher levels of RBC "survival" in the samples preserved with 200 μM spermine added to Alsever's solution. No effect of low (20 μM) spermine concentration on cell "survival" in low hematocrit blood samples was observed. However, as a component of the mixture, 20 μM spermine enhanced the positive effects of carnosine and phloretin.

Our data showed that phloretin (100 μM) as an additive to Alsever's solution improved RBC "survival" and blunted $[\text{Ca}^{2+}]_i$ increase in low- as well as in high-hematocrit samples after 36 days of storage. The reported effects of phloretin include antioxidant and glycation inhibitory effect [41]; the blockage of glucose (GLU1) transporter [43], that affects the cell metabolism and pH level; inhibition of Na^+/Li^+ exchanger [62]; $\text{Na}^+/\text{Li}^+-\text{PO}_4$ co-transporter [63]; AQP9 channels [44]; cyclic AMP-activated Cl^- channels [64]. The positive effect of phloretin on the stored cells survival could be due to its inhibitory effect on erythrocyte GLU1. Alsever's solution contains high ($\sim 114\text{ mM}$) glucose concentration. Low temperature storage slows down cell metabolism and glucose consumption. Excessive glucose penetration in the cells could result in HbA_{1c} formation and cell ageing. Partial blockage of GLU1 by phloretin [43, 65] may protect the cells against ageing and shrinkage. Decline in $[\text{Ca}^{2+}]_i$ in the cells stored in the presence of phloretin may relate to its inhibitory effect on AQP. Our recent data [38] showed that AQP9 channel contributes to the total cation (including Ca^{2+}) conductance in RBCs. However, phloretin, as a single additive to Alsever's solution, dramatically enhanced PS exposure (see Fig. 6) in the stored low hematocrit samples after the post- incubation.

In conclusion, the data suggested that only the three-component mixture of antioxidants carnosine, spermine and phloretin improved RBCs "survival" after the 36 days of storage and guaranteed reasonable levels of cell "survival" for both high and low hematocrit non-leukoreduced blood samples under the conditions modelling the transfusion of preserved cells (Table 1). However, the best results of RBC "survival" were obtained with low hematocrit blood samples, where the concentration of plasma and buffy coat cells was highly reduced. Thus, the mixture of the natural antioxidants (carnosine, spermine and phloretin) could be recommended, after additional experiments confirming low toxicity of the mixture for RBCs, for an improved version of Alsever's solution for low hematocrit non-leukoreduced whole blood or leukoreduced concentrated RBCs preservation.

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Disclosure Statement

The authors have no conflict of interest.

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