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Prolonged storage of thawed red blood cells

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Introduction. In modern transfusion practice, both in peacetime and in military conditions, red blood cells (RBCs) are widely used as the main component of donor blood. Cryopreserved red blood cells are considered the most safe and high-quality RBC-containing environment. However, the storage period of thawed RBCs after cryopreservation is limited to 24 hours, and significantly complicates their use. Therefore, extending the storage period of thawed RBCs is relevant for the blood service. Research objective: study the RBCs morphological state and functional completeness that were cryopreserved at -40°C and stored for 7 days at a temperature of +2°C - +4°C after thawing.

Materials and methods. The object of the study were RBCs that were cryopreserved at -40°C and stored for 7 days at a temperature of +2°C - +4°C after thawing. Deglycerolization of the thawed red blood cells, cryopreserved at -40°C, required three time washing by using reverse cytoagglomeration. Thawed RBCs were re-suspended in lactate-sucrose-phosphate solution. After RBC thawing and storage for 7 days (186 doses) in the suspension the following indicators were studied: free hemoglobin, extracellular potassium, adenosine triphosphate (ATP), 2,3-diphosphoglycerate (2,3-DPG), hematocrit, degree of hemoglobin affinity to oxygen (P50), viscosity coefficient, osmotic stability, electrophoretic mobility of erythrocytes. As well as the total number of cells lost and recovered.

Results. After storage for 7 days of suspension of thawed RBCs at a temperature of +2°C - +4°C indicators of free hemoglobin (0,62±0,02 g/l), extracellular potassium (2,7±0,3 mmol/l), hematocrit (0,4±0,02 l/l) were within normal limits. Osmotic resistance (0,46±0,02%), electrophoretic mobility (0,94±0,04 µm·cm·V-1·s-1) of RBCs, suspension viscosity factor (5,5±0,02mPa·s) did not exhibit changes in comparison with normal values. High levels of ATP indicators (3,0±0,2 µmol/gHb) and 2,3-DPG (10,5±1,3 µmol/gHb) were established. Indicator P50 (24,1±1,3 hPa) corresponded to low hemoglobin affinity for oxygen. After 7-day storage at +2°C - +4°C total cell loss was insignificant and amounted to 5,6±0,4%. High percentage of viable thawed RBCs 94,4±0,5% was shown.

Conclusions. Deglycerolization of thawed red blood cells, cryopreserved at -40°C, by reverse cytoagglomeration, as well as use of lactate-sucrose-phosphate solution for washed RBCs resuspending promote prolongation of thawed RBCs storage period up to 7 days at +2°C - +4°C in viable condition.

Keywords: Hematology, red blood cells, cryopreservation, thawing.
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Introduction

Improving blood system is a strategic task which affects provision of medical aid in peaceful time as well as during military operations [1,2,3]. Practical medicine makes extensive use of RBCs which are one of the donor blood major components. According to the modern data, the safest RBC-containing environment for recipient are thawed washed RBCs [4,5,6,7]. Cryobiology success allowed to consider cryopreservation to be an effective method of long-term blood cell storage [8,9,10,11,12]. There are two technological possibilities to cryopreserve RBCs. The first method is fast freezing and long-term RBCs storage at ultra-low temperature [13]. Practical implementation of this method requires complicated, cumbersome, expensive equipment with usage of liquid nitrogen. Another method is slow cooling and long-term storage of blood cell components (RBCs) at moderately low temperatures in electric refrigerators. The preference is given to more economic and simple methods of RBCs freezing and storage at moderately low temperatures [14,15]. But widespread clinical use of cryopreserved RBCs (irrespective of preservation method) is hindered by the storage period of thawed RBCs which is limited to 24 hours. Therefore, the issue of developing rational methods of thawed RBCs deglycerolization, as well as creation of special resuspending solutions to maintain RBCs functional properties during the post-thaw storage period, for the period exceeding 24 hours, is topical for the modern blood system [8,16,17,18]. Objective. To study the morphological state and functional capabilities of RBCs, that were cryopreserved at -40°C and stored after thawing for 7 days at a temperature of +2°C - +4°C.

Materials and Methods

The object of the study were cryopreserved RBCs at -40°C, which were stored after thawing for 7 days at a temperature of +2°C - +4°C. To obtain red blood cell component 1-day storage blood was used, obtained with informed consent from donors meeting all the procedural requirements under the Law of Ukraine [19]. In transfusion substances technology lab in PI IBP & TM AMSU, under aseptic conditions, plasma and white and platelet layers were removed by aspiration from hemocontainers (“Hemakon”, "RAVIMED" with hemopreservative CPDA-1 (Poland)) with whole donated blood. Free from plasma and white and platelet layers RBCs were transferred into sterile polymer cryocontainers CS 1000 and were supplemented in equal proportion (1:1) protective solution – cryopreservative, which contained: glycerol (State Pharmacopoeia of Ukraine, issue 1, p.355) – 791,2 g, dinatrii aethylendiamintetraacetas) (State Pharmacopoeia of Ukraine, issue 1, appendix 1.1, p.327) – 3,0 g, glucosum anhydricum (State Pharmacopoeia of Ukraine, issue 1, appendix 1.1, p.360) – 90,0 g and aqua ad injectabilia (State Pharmacopoeia of Ukraine, issue 1, appendix 1.1, p.307) - to 1000,0 ml. The final concentration of glycerol in the mix was 39.6%. After completion of glycerolization air was removed from the cryocontainer and the cryocontainer was sealed. RBCs, suspended in cryoprotective solution, were kept for 30 minutes at room temperature. After this cryocontainers with the mix of RBCs and cryopreservative were placed in low temperature electric refrigerators "Frigera" NZ280/75A or "Frigera" HZ700/50.2 (the Czech Republic) for freezing and storage up to 2 years at -40°C. 186 doses of RBCs were studied (table 1).

Table 1. The number of RBCs doses frozen at -40°C depending on their storage periods

<table>
<thead>
<tr>
<th>Red blood cells storage periods in frozen condition at temperature -40°C</th>
<th>The number of RBCs doses frozen at temperature -40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up to 6 months</td>
<td>36</td>
</tr>
<tr>
<td>From 6 months to 1 year</td>
<td>45</td>
</tr>
<tr>
<td>From 1 to 1,5 year</td>
<td>51</td>
</tr>
<tr>
<td>From 1,5 to 2 years</td>
<td>54</td>
</tr>
<tr>
<td>Total:</td>
<td>186</td>
</tr>
</tbody>
</table>

Thawing of the frozen RBCs was done in water bath at +38°C - +40°C. Suspension of the thawed RBCs was transferred into plasticized 1000,0 ml volume containers. To wash the RBCs from glycerol (deglycerolization process) the phenomenon of reverse cytoagglomeration of RBCs in low-ion environment, created due to sugars solutions. Glucose solutions of 30%, 10%, 5% were used. In the process of deglycerinization, thawed RBCs were added to the 30% glucose solution in the amount of 100 ml per 250 ml of the thawed RBCs mix was added to the mix of thawed RBCs and then it was mixed. In 2-3 minutes the mixture of RBCs and 30% glucose solution was mixed with 10% glucose solution in the amount equal to 4 volumes of frozen RBCs. After mixing there was RBCs agglomeration and their
sedimentation. The fluid above the sediment was removed. The agglomerated RBCs were once again mixed with 4 volumes of 10% glucose solution. After the RBCs settled, the washing fluid was removed. To prevent RBCs haemolysis and preserve in the mixture greater number of recovered cells the RBCs were mixed with 5% glucose solution, which was twice bigger in terms of volume than the volume of thawed RBCs. After sedimentation of agglomerated RBCs the fluid on top of the sediment was removed. Three times washing of the thawed RBCs promoted RBCs freeing from cryopreservative-glycerol and significant amount of free hemoglobin. Resuspending of agglomerated RBCs was done by adding to them two volumes of 0,9% saline solution, prepared according to the "Guideline" [20]. The latter procedure was applied to achieve complete deaggregation of thawed RBCs and to fully preserve their functional viability to prolong their storage term at temperature +2ºС - +4ºС. Deagglomerated RBCs were transferred to the polymer hemocontainers with 300,0 ml volume centrifuged at 1100 g during 10-12 minutes at +4ºC (centrifuge PC-6Мц, “Dastan”, Kyrgyzstan). The liquid that was above the sediment containing excess free hemoglobin was removed. Deglycerinized RBCs, were resuspended in the plasma-substituting lactate-sucrose-phosphate solution at the ratio of 1:1 or 1:½. Components of plasma-substituting solution: sucrose (PhEur, 10.0) - 7,0 g, dinatrii phosphas dodecahydricus (State Pharmacopoeia of Ukraine, issue 1, p.365) – 0,2 g, sodium hydrocitrate dibasic 1,5 liquor (State Pharmacopoeia of Ukraine, issue.2, p.493) - 0,1 g, sodium lactate (PhEur, 10.0) – 1,75 g and aqua ad iniectabilia (State Pharmacopoeia of Ukraine, issue 1, appendix 1.1, p.307) – up to 100,0 ml; рН solution – 6,8.

RBCs freezing, thawing, washing and resuspending procedures were performed under sterile conditions. Thawed deglycerolized RBCs, resuspended in plasma-substituting lactate-sucrose-phosphate solution, prior usage in the clinic were stored in the refrigerators (“Dnipro”, Ukraine) at temperature +2°С - +4°С during 7 days.

Immediately after thawing of RBCs, their deglycerolization, resuspension in lactate-sucrose-phosphate solution and on the 7th storage day at +2°С - +4°С the obtained RBCs were analysed with respect to the changes in the indicators’ dynamics:

- the content of free hemoglobin in the RBCs sediment using the ABX MICROS 60-OT analyzer (France);
- extracellular potassium level by flame photometry on FPL-1 device (Ukraine);
- Viscosity factor by formula:

\[
\mu = \frac{1.06 \cdot \text{RBCs susp.}}{\text{Tw}} \quad (\text{mPa.s})
\]

taking water density as \( p = 1 \text{ g/cm}^3 \),
where \( \mu \) – viscosity factor (mPa.s);
1,06 – blood density in g/cm3;
TRBCs susp. – time for 1 ml RBCs suspension outflow;
Tw - time for 1 ml of water outflow [20];
- corpuscular volume with ABX MICROS 60-OT analyzer (France);
- RBCs osmotic resistance [21];
- degree of hemoglobin affinity for oxygen (P50) [22];
- 2,3-diphosphoglycerate concentration (2,3-DPG) – indicator of RBCs oxygen delivery function [23];
- concentration of adenosine triphosphoric acid (ATP) - RBCs viability indicator [24].

- the number of red blood cells lost after all cryopreservation procedures (mixing RBCs with cryopreservative, freezing, thawing, washing, resuspending) and on the 7th day of post-thaw storage at +2°С - +4°С was determined by calculation method:

\[
\text{total cell loss (\%)} = \frac{\text{Hb free of washing solutions} + \text{Hb free of suspension}}{\text{Hb total}} \times 100,
\]

where \( \text{Hb total} \) – total hemoglobin in a frozen dose (g);
100 – factor of converting in percentage;
number of the recovered RBCs after all the cryopreservation procedures (mixing red blood cells with cryopreservative, freezing, thawing, washing, resuspending) and on the 7th post-thawing storage day at temperature +2°С - +4°С was calculated by formula:
number of the RBCs recovered = \( \frac{\text{Hb}_{\text{cellular}}}{\text{Hb}_{\text{cellular}} + \text{Hb}_{\text{free of the washing solution}}} \times 100 \)

where \( \text{Hb}_{\text{cellular}} \) – total hemoglobin of the entire dose of the washed RBCs concentrate (g);
\( \text{Hb}_{\text{free}} \) - free hemoglobin, dissolved in the entire volume of the washed RBCs (g);
100 – percentage conversion factor;

- RBCs electrophoretic mobility (EPM) – with "Opton" cytopherometer (Germany) under standard conditions [25]: current - 5 mA, voltage - 100 V, suspension fluid temperature - 25°C. Normal saline was taken as suspension fluid, buffered with phosphate buffer to pH 7,28-7,30. Time necessary for the cell to cover a certain distance (two squares of a grid net micrometer) was calculated with a stopwatch. In each specific case the speed of movement of 40-50 cells was counted.

Package “STATISTICA FOR WINDOWS 6.0” (Statsoft, USA) was used to provide statistic processing of the results. Difference probability between average indicators (p) in groups, which were compared, were established with Student’s t-test (t); difference probability between average values was taken as p<0,05.

Results
We conducted a study of stability and morphological completeness of cryopreserved RBCs at -40°C, which were stored after thawing for 7 days at a temperature of +2°C - +4°C. To achieve this, the intactness of their membranes, as well as the degree of destruction (hemolysis) was studied by the indicators of the free hemoglobin content, the concentration of extracellular potassium, electrophoretic mobility, the number of lost cells and the yield of restored cells. We evaluated the possibilities of engraftment of thawed RBCs by studying their osmotic stability in vitro.

In order to evaluate the viability, energy potential, the possibilities of the oxygen transport function, the ability to give oxygen to the tissues by thawed RBCs during the 7-day storage period at a temperature of +2°C - +4°C, we studied the content of ATP in RBCs (an indicator of viability, energy potential) and 2,3-DFG (an indicator of oxygen transport function), as well as the degree of their hemoglobin affinity to oxygen (an indicator of P\textsubscript{50}).

Table 2. Indicators of the morphological state and functional capabilities of cryopreserved RBCs at -40°C immediately after thawing and on the 7th day of storage at a temperature of +2°C - +4°C (n = 186)

<table>
<thead>
<tr>
<th>Indicators under study</th>
<th>Statistical indicators</th>
<th>Reference values</th>
<th>Immediately after thawing</th>
<th>7\textsuperscript{th} storage day at +4 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit, l/l</td>
<td>M± m p</td>
<td>0,36-0,46 males</td>
<td>0,39 ± 0,01</td>
<td>0,40 ± 0,02, &gt; 0,05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,41-0,53 females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free hemoglobin, g/l</td>
<td>M ± m p</td>
<td>0,20-0,70</td>
<td>0,35 ± 0,03</td>
<td>0,42 ± 0,02, &gt; 0,05</td>
</tr>
<tr>
<td>Extracellular potassium, mmol/L</td>
<td>M ± m p</td>
<td>1,2-8,9</td>
<td>1,7 ± 0,2</td>
<td>2,3 ± 0,4, &gt; 0,05</td>
</tr>
<tr>
<td>ATP, mcmol/g Hb</td>
<td>M ± m p</td>
<td>2,0 – 4,0</td>
<td>3,0 ± 0,2</td>
<td>2,5 ± 0,2, &gt; 0,05</td>
</tr>
<tr>
<td>2,3- DPG, mcmol/g Hb</td>
<td>M ± m p</td>
<td>6,0 – 12,0</td>
<td>11,2 ± 1,0</td>
<td>10,5 ± 1,3, &gt; 0,05</td>
</tr>
<tr>
<td>RBCs osmotic resistance, % sodium chloride</td>
<td>M ± m p</td>
<td>0,40</td>
<td>0,42 ± 0,02</td>
<td>0,46 ± 0,02, &gt; 0,05</td>
</tr>
<tr>
<td>RBCs electrophoretic mobility, µm·cm·V\textsuperscript{-1}·s\textsuperscript{-1}</td>
<td>M ± m p</td>
<td>1,088</td>
<td>0,945 ± 0,020</td>
<td>0,935 ± 0,040, &gt; 0,05</td>
</tr>
<tr>
<td>Suspension viscosity factor, mPa·C</td>
<td>M ± m p</td>
<td>5,9</td>
<td>5,40 ± 0,20</td>
<td>5,50 ± 0,16, &gt; 0,05</td>
</tr>
<tr>
<td>P\textsubscript{50}, hPa</td>
<td>M ± m p</td>
<td>34,7</td>
<td>25,5±1,6</td>
<td>24,1±1,3, &gt; 0,05</td>
</tr>
</tbody>
</table>

Note: p – credibility of the difference of thawed RBCs indicators under research on the 7th storage day at +4 °C in comparison with indicators directly after thawing.
As it is shown by data in table 2 in the suspension of the thawed RBCs on the 7th storage day at +2°C - +4°C free hemoglobin content was normal and did not exceed the transfusion value of 1, 2 g/l acceptable in the clinic, which demonstrated RBCs resistance, stability, no destruction, haemolysis.

Extracelluar potassium level indicator on the 7th day of storage at +2°C - +4°C fluctuated within normal values (table 2). It showed that a RBC membrane was preserved.

An important criterion characterizing the effectiveness of the washing and resuspension process is the value of cell loss and cell recovery. After 7 days of storage of thawed RBCs at temperature +2°C - +4°C, the total cell loss was insignificant and amounted to 5.6 ± 0.4%. It was established that the use of deglycerolization of thawed RBCs by reverse cytoagglomeration, as well as the use of lactate-sucrose-phosphate solution for their resuspension ensured a high yield of 94.4 ± 0.5% of recovered viable cells after their 7-day storage at +2°C - +4°C.

To determine the integrity of thawed RBCs cellular membrane depending on the storage at +2°C - +4°C their electrophoretic mobility was studied (EPM). The research has shown that on the 7th day of storage EPM of thawed RBCs was within normal values (table 2). The findings have shown no thawed RBCs membrane changes during 7-day storage at +2°C - +4°C.

Taking into account the fact that RBCs osmotic resistance is a test which can be used to in vitro evaluate RBCs acceptance in patient’s blood flow, we have researched the changes in this indicator depending on the storage period of RBCs at +2°C - +4°C. The studies have shown that on the 7th storage day high RBCs osmotic resistance values were observed (table 2). High osmotic resistance of thawed RBCs during 7-day storage period at +2°C - +4°C is explained by the fact that during washing process unstable cells, which are in pre-haemolytic stage and, consequently, potentially lack viability, are removed from thawed RBCs suspension.

On the 7th day of thawed RBCs storage at a temperature of +2°C - +4°C, no significant changes were observed in the viscosity coefficient of the suspension, as well as the hematocrit index (table 2). This contributed to the preservation of the normal shape of RBCs and the intact state of their membranes.

Discussion
The content of ATP and 2,3-DPG, indicators of viability, energy potential, oxygen transport function, in thawed RBCs on the 7th day of storage were within the normal range (table 2). The P50 indicator, which reflects the degree of hemoglobin affinity to oxygen, decreased on the 7th day of storage of thawed RBCs at a temperature of +2°C - +4°C (table 2), which indicated a decrease in the degree of hemoglobin affinity to oxygen and simplification of its return to the tissues. Thus, the normal level of ATP and 2,3-DPG and a decrease in the P50 level indicated the viability, high energy potential, and high oxygen transport capabilities of thawed RBCs on the 7th day of their storage at a temperature of +2°C - +4 °C.

In conclusions, the obtained research results showed that the use of deglycerination of thawed RBCs, cryopreserved at -40°C, by the reverse cytoagglomeration method, as well as the use of plasma-substituting lactate-sucrose-phosphate solution for resuspension of washed RBCs make it possible to extend their storage period at a temperature of +2°C - +4 °C from 24 hours to 7 days in a functionally complete state, which can contribute to the widespread implementation of RBC cryopreservation methods in the state blood service.

References


