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USE OF LACTULOSE IN THE COMPOSITION OF BLOOD CELL CRYOPRESERVATIVES

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Introduction. Cryopreservation allows for long-term conservation of biomaterials. The insufficient efficacy of available cryopreservatives and the toxicity of a number of cryocomponents renders the search for low-toxic biocompatible cryoagents highly relevant.

Objective. Assessment of morphological and functional features of blood cells in a lactulose-based cryopreservative for storing whole blood at moderately low temperatures (minus 20 °C) using leukocyte, platelet, and erythrocytes parameters.

Materials and methods. The study was conducted using peripheral venous blood of 30 female donor volunteers aged 18–23 years. Samples of peripheral venous blood were stabilized by 3-substituted potassium salt of ethylenediaminetetraacetic acid. The cryopreservative was prepared using a 0.9 % sodium chloride solution to maintain the isotonic concentration. Glycerin and dimethyl sulfoxide were used as cell-penetrating cryoprotectors; lactulose disaccharide was used as a non-penetrating cryoprotector. The composition of the obtained cryopreservative was optimized by varying the mass fractions of the components. Clinical blood tests were performed using a Gemalite 1270 automatic hematology analyzer. A computer cytomorphometric study was performed in the MEKOS-C2 hardware and software environment.

Results. The conservation of blood samples using the developed cryopreservative for 24 h at a temperature of minus 20 °C increased the percentage of preserved leukocytes, erythrocytes, and platelets to 88.6±0.41 %, 92.1±0.31 %, and 91.4±0.52 %, respectively. The blood cells retained their physiological activity after thawing compared to blood samples stored at room temperature.

Conclusions. The morphological and functional safety of blood cells in samples stored with the developed cryopreservative was revealed after 24 h of storage at minus 20°C. The advantages of this cryopreservative include the possibility of its long-term storage without loss of cryoprotective properties, stabilizing blood cells to the effects of sub-moderate low temperatures of minus 20 °C, the use of non-toxic lactulose disaccharide that does not penetrate into the cell. The developed cryopreservative proves effective in freezing conditions at minus 20 °C, being affordable in terms of cost (all components are manufactured in the Russian Federation). Further research in this direction will contribute to the development of safer blood donation approaches and reducing complications during transfusion of blood components.

Keywords: cryopreservation; cryoprotectors; erythrocytes; leukocytes; platelets; lactulose; morphofunctional properties

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ИСПОЛЬЗОВАНИЕ ЛАКТУЛОЗЫ В СОСТАВЕ КРИОКОНСЕРВАНТА ДЛЯ СОХРАНЕНИЯ КЛЕТОК КРОВИ

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Введение. Криоконсервация позволяет длительно сохранять биоматериал, однако существует ряд проблем, связанных с недостаточной эффективностью криоконсервантов и токсичностью ряда криокомпонентов, в связи с чем актуален поиск низкотоксичных биосовместимых криоагентов.

Цель. Оценка морфофункциональных особенностей форменных элементов крови в криоконсерванте с лактулозой на основании показателей лейкоцитарных, тромбоцитарных и эритроцитарных параметров для хранения цельной крови при умеренно низкой температуре (–20 °С).

Материалы и методы. Исследование проведено на 30 добровольцах-донорах женского пола в возрасте 18–23 лет. Объект исследования — периферическая венозная кровь, стабилизированная 3-замещенной калиевой солью этилендиаминтетрауксусной кислоты. При приготовлении модельного криоконсерванта был использован 0,9 % раствор хлорида натрия для поддержания изотонической концентрации. В качестве криопротекторов, проникающих в клетку, использовали глицерин и диметилсульфоксид, в качестве не проникающего — дисахарид лактулозу. Оптимизация состава криоконсерванта проводилась за счет варьирования массовых долей компонентов. Общий анализ крови выполняли на автоматическом гематологическом анализаторе «Гемалайт 1270». Компьютерное цитоморфометрическое исследование проводили на аппаратно-программном комплексе «МЕКОС-Ц2».

Результаты. В ходе исследования в условиях сохранения образцов крови с применением разработанного криоконсерванта после 24 ч хранения при температуре –20 °С увеличивался процент сохранности лейкоцитов, эритроцитов, тромбоцитов (88,6 ± 0,41, 92,1 ± 0,31, 91,4 ± 0,52% соответственно) с сохранением форменных элементов крови в физиологически активном состоянии после оттаивания по сравнению с образцами крови, сохранявшимися при комнатной температуре.

Выводы. Выявлена морфологическая и функциональная сохранность форменных элементов крови в образцах с применением разработанного криоконсерванта после 24 ч хранения при температуре –20 °С. Преимущества данного криоконсерванта: возможность его длительного хранения без потери криопротекторных свойств, обеспечение криопротектором стабилизации форменных элементов крови к воздействию субумеренно

низкой температуры –20 °С, применение нетоксичного дисахарида лактулозы, не проникающего внутрь клетки. Разработанный криоконсервант является эффективным в условиях замораживания при –20 °С и доступным (все компоненты производятся на территории Российской Федерации). Исследования в данном направлении позволят более эффективно использовать аутодонорство во избежание ряда осложнений при трансфузии компонентов крови.

Ключевые слова: криоконсервация; криопротекторы; эритроциты; лейкоциты; тромбоциты; лактулоза; морфофункциональные свойства

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INTRODUCTION

Preservation of blood cells outside their native environment is a relevant task in practical healthcare. Cryopreservation is indented for long-term storage of biosamples without changes in their cellular structure and functional activity. The use of liquid nitrogen requires bulky and expensive equipment, as well as regular replenishment of expendable materials, which affects the cost of material storage and transportation of samples [1]. Therefore, effective cryopreservatives for the temperature range from –20°C to –80° are in high demand. A distinctive disadvantage of cryopreservation consists in the possible destruction of cell membranes due to insufficient efficiency of existing cryopreservatives [2]. At the same time, cryoagents should not only possess low toxicity [3, 4], but also prevent the loss of phenotypic and functional properties of the biomaterial. Therefore, the search for low-toxic biocompatible cryoagents represents a highly relevant research task [5].

The highest results are achieved using combined cryopreservatives, including endo- and exocellular cryoprotectants [6]. The toxic effect of cryosystems can be reduced by introducing lipids, proteins, carbohydrates [7–10], amino acids [11], and polyatomic spirits [12] as cryoagents of natural origin. Low-molecular carbohydrates are a promising class of cryoprotectors [13]. Disaccharide trehalose has a pronounced protective effect on cell membranes [14]. Mixtures of sugars and polyols are considered as a natural eutectic system [15]. At present, the literature lacks experimental data on effects of a number of carbohydrates, disaccharide lactulose in particular, on cells when used as cryoprotectants. Lactulose is a safe compound for all age groups, including children under one year of age [16]. In a study of lactulose, no data on toxic, teratogenic, and mutagenic effects were obtained in clinical studies involving humans [17]. At the same time, protective properties of lactulose on microorganisms during their freezing were noted [18, 19].

The need for whole blood and its components is constantly increasing [20]. For analytical purposes, fresh whole blood samples are preferred; however, this implies the necessity of rapid analysis after collection and the limited number of repeat tests that can be performed without additional blood collection [21]. Although the experience of cryopreservation in the field of capillary blood with subsequent analysis by cytometry was described in [22], no similar data on cryopreservation of venous blood can be found. Thus, it appears relevant to analyze the morphological and functional parameters of cryopreserved whole venous blood with the purpose of determining the possibility of increasing the storage duration of stabilized blood samples without changes in analytical techniques and blood parameters.

In this study, we evaluate the morphological and functional features of blood cells preserved with a lactulose-based cryopreservative using leukocyte, platelet, and erythrocytes parameters for whole blood storage at moderately low temperatures (–20°C).

MATERIALS AND METHODS

The study was conducted using blood samples from 30 healthy female donors aged 18–23 years. All participants were distributed according to the phase of the menstrual cycle (follicular phase). Inclusion criteria for the study were the absence of chronic diseases in the period of exacerbation, bad habits, and visible signs of allergic or infectious diseases. All female participants signed informed consent to participate in the study. The research object was peripheral venous blood stabilized with 3-substituted potassium salt of ethylenediaminetetraacetic acid (K3 EDTA) in vitro. Venous blood samples in the amount of 15 mL were collected once in the morning from the elbow vein into specialized vacuum tubes for hematological studies with K3 EDTA.

Experimental samples were divided into three groups. The control group included 10 blood samples, in which the characteristics and parameters of blood elements were investigated at a temperature of +20 ± 1.0°C. Experimental group I included 10 blood samples, in which a model cryopreservative was introduced beforehand. These samples were examined after 4 h, at a temperature of +20 ± 1.0°C. Experimental group II included 10 blood samples, into

which the model cryopreservative was introduced, followed by introducing the samples into a state of cold anabiosis at –20 ± 1.0°C for 24 h to evaluate the preservation of samples after a single freeze/thaw cycle. The samples were then unfrozen for a subsequent examination of the characteristics and parameters of blood elements at +20 ± 1.0°C.

When preparing a model cryopreservative, a 0.9% sodium chloride solution was used to maintain isotonic concentration. Glycerol and dimethyl sulfoxide (DMSO) were used as cell-penetrating cryoprotectants, while disaccharide lactulose was used as a non-penetrating cryoprotectant. The cryopreservative composition was optimized by varying the mass fractions of the components. The final composition of the model cryopreservative had the following ratios of components: 20% of glycerol (analytic grade), 10% of DMSO (chemically pure), 2.5% of lactulose (*Lactusan* trade mark according to TU 9229-004-53757476-04), and isotonic (0.9%) sodium chloride solution up to 100%. The prepared solution was autoclaved (without DMSO) at 1.2 atm for 30 min and stored in a refrigerator at 2–4°C. DMSO is not subject to autoclaving due to its oxidizing in air, which leads to its decomposition into compounds that increases the toxicity of the composition. Sterilization of DMSO was carried out by the sterilizing filtration method with the subsequent storage of DMSO in glass sterile tubes at –10°C. Thawing of DMSO was performed in a UT-4334 water bath (ULAB, Russia) at 37 ± 1°C. DMSO was added to the prepared sterile model cryopreservative immediately before freezing the experimental samples.

The model cryopreservative in the amount of 500 μL was added to the samples of experimental groups I and II using volume changer pipettes with the 2:1 ratio of venous blood/cryopreservative by volume. The tubes were sealed with stoppers, and the contents were stirred for 10 min using an orbital shaker (PSU-10i, Latvia). Following 4 h, group I samples were examined at +20 ± 1.0°C. Group II samples were placed in the freezing chamber of an electric freezer with a temperature of –20 ± 1.0°C and kept for 24 h. Subsequently, the samples were thawed in a water bath UT-4334 (ULAB, Russia) in manual mode at a temperature of +37 ± 1°C for 1 min. Further, the characteristics and parameters of blood elements were investigated at +20 ± 1.0°C. A computer cytomorphometric study of blood cells was performed in the MECOS-C2 hardware and software environment (Medical Computer Systems, Russia). *In vitro* diagnostic tests of the total blood count (TBC) were performed using a Hemalight 1270 automatic hematological analyzer (Dixion, Russia).

The obtained results were processed using the IBM SPSS Statistic 23.0 software statistical package (IBM Corp., Armonk, NY, USA). The distribution of the studied parameters was evaluated using the Shapiro–Wilk W-criterion. The level of statistical significance of intergroup differences when the distribution of indicator values conformed to the law of normal distribution was evaluated using Student's t-criterion for unrelated samples. For indicators with non-normal distribution, the nonparametric Mann–Whitney U-criterion was applied. For indicators with normal distribution, the mean (*M*), error of mean (*m*), and standard deviation (δ) were calculated. Intergroup differences were considered statistically significant at $p \leq 0.05$.

RESULTS

We analyzed the leukocyte dynamics in experimental and control groups (at +20 ± 1.0°C in experimental group I with the cryopreservative and at –20 ± 1.0°C in experimental group II with the cryopreservative). The corresponding data are presented in Table 1.

When analyzing leukocytic indices in both experimental groups, compared to the control, a statistically significant decrease in the number of leukocytes was noted. The most pronounced decrease was observed experimental group II, $(3.94 \pm 0.87) \times 10^9$/L ($p < 0.01$), against $(4.55 \pm 0.83) \times 10^9$/L in experimental group I and $(5.60 \pm 0.92) \times 10^9$/L in the control, respectively. Meanwhile, the value of mean cells was significantly increased in both group I and group II compared to the control, indicating a decrease in leukocyte cell recognition by the blood analyzer. Despite significant intergroup differences, this index ranged within physiologic reference values. The percentage of leukocyte preservation in blood samples with the cryopreservative under study (temperature +20 ± 1.0°C) was 81 ± 0.89%, and 88.6 ± 0.41% when exposed to negative temperatures (–20 ± 1.0°C).

Computer morphometry allows mathematical characteristics of the cell population to be obtained, at the same time as providing an opportunity to estimate the activity of intracellular processes [23]. To assess the functional state of cells, we analyzed computerized cytomorphometry of leukocytes in the control group and experimental groups (at +20 ± 1.0°C in experimental group I with the cryopreservative and at –20 ± 1.0°C in experimental group

Table 1. Leukocyte indices of total blood count (TBC) in the study groups

Total blood counts	Control group $n = 10$	Experimental group I $n = 10$	Experimental group II $n = 10$
Leukocytes, $\times 10^9$/L	5.60 ± 0.92	4.55 ± 0.83*	3.94 ± 0.87*
Lymphocytes, %	38.31 ± 4.21	25.70 ± 3.14	25.70 ± 2.17
Granulocytes, %	56.20 ± 5.23	51.10 ± 4.24	51.10 ± 3.21
Percentage of medium-sized cells, %	6.20 ± 1.43	10.20 ± 1.47*◆	9.20 ± 1.23*◆

Table prepared by the authors based on their own data

Note: data are presented as mean value and standard error of the mean (*M* ± *m*);
* — statistically significant difference between control and experimental groups ($p < 0.01$)
◆ — statistically significant difference between experimental groups I and II ($p < 0.01$)

II with the cryopreservative). The corresponding data are presented in Table 2.

An analysis of the computer cytomorphometry data of leukocytes showed that in group II (samples with the cryopreservative frozen at –20 ± 1.0°C), the optical density of cytoplasm significantly increased up to 1.03 ± 0.01 c.u. both in comparison with the control, 0.66 ± 0.01 c.u., and experimental group I, 0.50 ± 0.01 c.u. This might indicate the increased permeability of endocellular cryoagents into the cell.

Erythrocyte parameters were also analyzed in the control group and experimental groups (at +20 ± 1.0°C in experimental group I with the cryopreservative and at –20 ± 1.0°C in experimental group II with the cryopreservative). The corresponding data are presented in Table 3.

When performing the total blood analysis, values indicating changes in quantitative and calculated indices were obtained. Thus, a significant decrease in the level of total blood hemoglobin in both experimental groups (119.25 ± 4.27 g/L and 111.64 ± 4.42 g/L) compared to the control group was observed. In addition, a decrease in the average hemoglobin content in erythrocytes in both experimental groups, compared to the control, was noted. These indicators showed a decreasing trend. This indirectly points to pre-dilution of the studied sample; however, it can be said that the cryopreservative does not lead to critically significant changes in blood components at room temperature. The recorded decrease in both total hemoglobin and its average content in cells indicates the processes of increased transmembrane permeability, while these indicators did not exceed the reference range of physiologically acceptable values in the experimental groups.

The percentage of erythrocyte preservation in blood samples with the cryopreservative applied (temperature +20 ± 1.0°C) was 89 ± 0.2%, and 92.1 ± 0.31% when exposed to negative temperatures (–20 ± 1.0°C). The conducted computer cytomorphometry of erythrocytes in both experimental groups, compared to the control, revealed a significant increase in the cell area in group I (15 ± 1.22 μm^2) and group II (22 ± 1.45 μm^2), compared to 14 ± 2.1 μm^2 in the control. The value of mean cell diameter tends to increase, comprising 6.11 ± 0.91 μm in group I and 9.04 ± 1.34 μm

Table 2. Difference indices of computerized cytomorphometry of leukocytes in the study groups

Parameter	Control group *n* = 10	Experimental group I *n* = 10	Experimental group II *n* = 10
Cell area, μm^2	70.00 ± 4.16	59.00 ± 5.23	60 ± 5.92
Cell form factor, %	14.11 ± 5.12	18.01 ± 3.84	16.7 ± 4.33
Cell polarization index, %	0.16 ± 0.01	0.31 ± 0.01	0.14 ± 0.01
Optical density of cytoplasm, conventional units	0.66 ± 0.01	0.50 ± 0.01*♦	1.03 ± 0.01*♦
Nucleus area, μm^2	52.00 ± 5.32	40.00 ± 4.88*♦	38 ± 4.41*♦
Core form factor, %	14.30 ± 3.41	13.10 ± 2.88	11.4 ± 3.23
Core polarization, %	0.06 ± 0.01	0.02 ± 0.01	0.25 ± 0.01
Nuclear cell ratio, %	0.74 ± 0.01	0.68 ± 0.01	0.63 ± 0.01
Share of core complementation, %	0.04 ± 0.01	0.02 ± 0.01	0.05 ± 0.01

Table prepared by the authors using their ow data

Note: data are presented as mean value and standard error of the mean (*M* ± *m*);
* — statistically significant difference between control and experimental subjects ($p < 0.01$)
♦ — statistically significant difference between the 1st and 2nd experimental groups ($p < 0.01$)

Table 3. Erythrocytic parameters of TBC in the experimental groups

Total blood counts	Control group *n* = 10	Experimental group I *n* = 10	Experimental group II *n* = 10
Erythrocytes, ×10^{12}/L	4.62 ± 0.24	4.08 ± 0.22	3.75 ± 0.41
Hemoglobin, g/L	131 ± 5.34	119.25 ± 4.27*	111.64 ± 4.42*♦
Average erythrocyte volume, fl	86.3 ± 4.36	84.3 ± 4.11	81.7 ± 3.47
Hematocrit, %	39.3 ± 1.55	35.4 ± 1.45	32.2 ± 1.27
Average hemoglobin content in erythrocytes, pg	28.5 ± 0.3	26.2 ± 1.47*	23.7 ± 1.25*♦
Erythrocyte hemoglobin saturation, g/L	336 ± 29.74	311 ± 23.37	305 ± 19.89
Degree of erythrocyte size deviation, %	12.2 ± 1.65	11.7 ± 1.44	10.5 ± 1.47

Table prepared by the authors using their own data

Note: data are presented as mean value and standard error of the mean (*M* ± *m*);
* — statistically significant difference between control and experimental groups ($p < 0.01$)
♦ — statistically significant difference between the 1st and 2nd experimental groups ($p < 0.01$)

Table 4. Difference indices of computerized cytomorphometry of erythrocytes in the study groups

Parameter	Control group *n* = 10	Experimental group I *n* = 10	Experimental group II *n* = 10
Cell area, μm²	14 ± 2.1	15 ± 1.22*	22 ± 1.45*◆
Average cell diameter, μm	6.11 ± 0.91	7.01 ± 1.12	9.04 ± 1.34
Form factor, %	0.16 ± 0.01	0.31 ± 0.01	0.33 ± 0.01
Polarization, %	0.66 ± 0.01	0.50 ± 0.01	0.47 ± 0.01
Integral optical density (red), μm²	52 ± 2.18	40 ± 2.25	38 ± 1.43
Integral optical density (green), μm²	14.3 ± 2.15	13.1 ± 1.49	12.1 ± 1.43
Integral optical density (blue), μm²	0.06 ± 0.01	0.02 ± 0.01	0.02 ± 0.01

Table prepared by the authors using their own data

Note: data are presented as mean value and standard error of the mean (*M* ± *m*);
* — statistically significant difference between control and experimental groups ($p < 0.01$)
◆ — statistically significant difference between the 1st and 2nd experimental groups ($p < 0.01$)

Table 5. Platelet TBC indices in the study groups

Total blood counts	Control group *n* = 10	Experimental group I *n* = 10	Experimental group II *n* = 10
Thrombocytes, ×10⁹/L	230.08 ± 6.31	198.60 ± 5.36*	181.5 ± 5.71*◆
Average platelet volume, fl	8.24 ± 1.31	7.56 ± 1.47	6.13 ± 1.54
Thrombocrit, %	2.23 ± 0.65	2.10 ± 0.74	2.03 ± 0.14
Large thrombocyte ratio, %	17.38 ± 3.31	21.24 ± 2.36	20.12 ± 3.51

Table prepared by the authors using their own data

Note: data are presented as mean value and standard error of the mean (*M* ± *m*);
* — statistically significant difference between control and experimental groups ($p < 0.01$)
◆ — statistically significant difference between the 1st and 2nd experimental groups ($p < 0.01$)

in group II, compared to 7.01 ± 1.12 μm in the control. The increase in both the area and diameter of erythrocytes indicates a possible increase in the permeability of the applied cryoagent inside the cell. The corresponding data are presented in Table 4.

The conducted comparative analysis of platelet indices found the number of platelets to decrease significantly. Thus, in groups I and II, this value was (198.60 ± 5.36)×10⁹/L and (181.5 ± 5.71)×10⁹/L, respectively, compared to (230.08 ± 6.31)×10⁹/L in the control. The decrease in the number of cells was most likely caused by the increase in the volume of the studied sample against the background of cryopreservative application. At the same time, the coefficient of large platelets in both experimental groups in comparison with the control group showed an increasing trend (see Table 5).

The percentage of platelet preservation in blood samples with the cryopreservative applied (temperature +20 ± 1.0°C) was 86.2 ± 0.31% and 91.4 ± 0.52% when exposed to negative temperatures (–20 ± 1.0°C). Presumably, the cryopreservative activates platelets to some extent, which is accompanied by their increase. However, all the studied parameters were within the range of acceptable physiological values. Therefore, we observed a reaction of platelet adaptation to the impact of a foreign component, i.e., cryopreservative.

The computer cytomorphometry of platelets in the experimental groups found a statistically significant increase in the cell area compared to the control. This parameter was more pronounced in the samples of group I (with the cryopreservative applied) at the level of 11.85 ± 1.15 μm², compared to 9.12 ± 1.12 μm² in group II (with the cryopreservative applied and freezing). It should be noted that despite significant intergroup differences, this index reflects the platelet area activity with subsequent adaptation after freezing. The marked changes were within the range of physiologic reference values. The corresponding data are presented in Table 6.

DISCUSSION

The conducted comparative analysis of TBC indices in the experimental groups with the cryopreservative (regardless of temperature conditions of blood sample preservation) established a reliable decrease in the total number of leukocytes. This finding might be related to changes in the ratio of liquid part of blood and blood elements under the conditions of cryopreservative application, as well as to the decrease in leukocyte cell recognition by the blood analyzer. At the same time, a reliable increase in the percentage of medium cells in samples with the preservation temperature of –20 ± 1.0°C was observed. It might be connected with changes in the leukocyte morphology, indicated by changes in the optical density of leukocyte cytoplasm revealed by computer cytomorphometry. These changes may also be associated with the action of the cryopreservative;

Table 6. Computerized cytomorphometry of platelets in the study groups

Parameter	Control group $n = 10$	Experimental group I $n = 10$	Experimental group II $n = 10$
Cell area, μm^2	7.70 ± 1.23	11.85 ± 1.15*	9.12 ± 1.12*♦
Min. diameter, μm	2.65 ± 0.15	2.83 ± 0.54	2.72 ± 0.71
Max. diameter, μm	4.03 ± 0.43	4.94 ± 0.33	4.81 ± 0.25
Average diameter, μm	3.44 ± 0.27	3.92 ± 0.78	3.88 ± 0.31
Form factor, %	12.90 ± 2.10	14.87 ± 2.88	13.10 ± 1.97

Table prepared by the authors using their own data

Note: data are presented as mean value and standard error of the mean ($M \pm m$);
* — statistically significant difference between control and experimental groups ($p < 0.01$)
♦ — statistically significant difference between the 1st and 2nd experimental groups ($p < 0.01$)

however, the samples subjected to freezing showed the stability of the indicators.

The comparison of erythrocyte indices in the groups with cryopreservative application, both under positive temperatures and during freezing of samples, revealed a significant decrease in the total blood hemoglobin and average hemoglobin content in erythrocytes. However, statistically significant changes in erythrocyte concentration were not revealed, which indicates the effectiveness of the applied cryoprotectant. At the same time, according to the computer cytomorphometry data, the increase in the erythrocyte cell area and the increasing trend of the average cell diameter and erythrocyte shape factor in the samples with cryopreservation and subsequent freezing, confirms the transmembrane penetration of cryoagents inside the cells.

The dynamics of platelet indices showed the platelet concentration to statistically significantly decrease in cryopreserved blood (+20 ± 1.0°C), which is associated with changes in the ratio of cells to the liquid part of the blood. A more pronounced decrease in this index was noted in the samples preserved under negative temperatures, compared to group I (with the cryopreservative at a temperature of +20 ± 1.0°C), while its changes did not exceed the limits of physiological norm. Thus, this blood preservation method can be used in the formation of a cryopreserved bank of platelet hemoconcentrate. The conducted computer cytomorphometry of platelet cells demonstrated a statistically significant increase in cell area. This increase might be explained by the fact that platelets trigger activation processes in response to contact with a foreign agent in the form of cryopreservative. At the same time, in blood samples of group II, also with the cryopreservative but during freezing, we registered a less pronounced increase in the platelet area. This can be explained by the onset of adaptation processes to the cryopreservative when exposed to negative temperatures; however, this observation requires further elucidation.

During preservation of blood samples with the developed cryopreservative for 24 h of storage at –20°C, the percentage of preserved blood cells, i.e., leukocytes, erythrocytes, and platelets comprised 88.6 ± 0.41%, 92.1 ± 0.31%, and 91.4 ± 0.52%, respectively. Importantly, the blood cells maintained their physiological activity after thawing, compared to the blood samples preserved at room temperature. Similar experiments conducted by Kiryanova et al. using a Cryosin solution as a cryopreservative showed the percentage of preserved erythrocytes in the amount of 83.8 ± 4.09% [24]. Works by Isaeva et al. aimed at assessing the viability of nucleus-containing cells in leukoconcentrates at the stages of their obtaining and freezing achieved the level of 86.7% cells in a viable state [25]. The research team of Vetoshkin et al. conducted studies by freezing of donor blood platelets using a combined cryopreservative and obtained the preservation of their functional activity at a level of 63.5–88.8% [26]. Whole blood cells after daily storage at –40°C were also studied, with the morphological and functional preservation of cells amounting to 85.3 ± 0.30% for erythrocytes, 75 ± 0.71% for platelets, and 90.1 ± 0.91% for leukocytes compared to the values registered before freezing [27].

Shirokikh et al. studied the effect of polysaccharide fractions on cryopreserved human venous blood and determined a decrease in the osmolarity of human blood from 281 to 149 mOsm/L. This result was attributed to the interaction of functional groups of polysaccharides with osmotically active substances of blood plasma, leading to a decrease in the osmolarity of the medium and acceleration of water crystallization [28]. Lactulose (as well as other disaccharides) belongs to non-penetrating cryoprotectants. Such compositions create osmotic pressure, which causes dehydration of cells and reduces the degree of ice formation inside cells. The cryo-effect of lactulose is apparently similar to that of trehalose disaccharide, which acts as an inhibitor of ice crystal growth during freezing and recrystallization of ice during thawing, forming a highly viscous glass-like state [29]. Nevertheless, the cryoprotective effect of lactulose has not yet been fully determined, requiring further investigation.

The main results of our study are protected by the patent for invention [30]. The advantages of using the developed cryoprotectant include the possibility of long-term storage without loss of cryoprotective properties and stabilization of blood cell elements under sub-zero temperatures of –20°C. The preparation of cryoprotectant does not require bulky and expensive equipment, as well as toxic components. Disaccharide lactulose is non-toxic compound, not penetrating inside the cell.

CONCLUSION

Our studies have confirmed the possibility of successful morphological and functional preservation of blood cells with the proposed cryopreservative after 24 h of storage

at –20°C. The developed cryopreservative is affordable (all components are produced in the Russian Federation) and effective for freezing blood samples at –20°C. This extends the range of current cryopreservatives applied for analysis of morphological and functional parameters of frozen whole blood samples in large-scale studies and field medicine, for storage of biomaterial in long-term expeditions and remote areas. The works carried out in this direction contribute to the development of effective blood donation approaches, allowing complications in transfusion of blood components to be mitigated.

In our opinion, lactulose deserves further study as a cryocomponent for ensuring the safety of blood cells during long-term storage periods.

References

1. Hidi L, Komorowicz E, Kovács GI, Szeberin Z, Garbaisz D, Nikolova N, et al. Cryopreservation moderates the thrombogenicity of arterial allografts during storage. *PLoS One.* 2021;16(7): e0255114. https://doi.org/10.1371/journal.pone.0255114
2. Bojic S, Murray A, Bentley BL, Spindler R, Pawlik P, Cordeiro JL, et al. Winter is coming: the future of cryopreservation. *BMC Biol*. 2021;19(1):56. https://doi.org/10.1186/s12915-021-00976-8
3. Awan M, Buriak I, Fleck R, Fuller B, Goltsev A, Kerby J, et al. Dimethyl sulfoxide: a central player since the dawn of cryobiology, is efficacy balanced by toxicity? *Regenerative medicine*. 2020;15(3):1463–91. https://doi.org/10.2217/rme-2019-0145
4. Liu X, Pan Y, Liu F, He Y, Zhu Q, Liu Z, et al. A review of the material characteristics, antifreeze mechanisms, and applications of cryoprotectants (CPAs). *Journal of Nanomaterials*. 2021;(2):1–14. https://doi.org/10.1155/2021/9990709
5. Zaikina EV, Goncharova AS, Pozdnyakova VV, Pandova OV, Przhedetskiy YV, Volovik VG, et al. Review of modern methods of cryopreservation of various types of biological material. *Modern problems of science and education.* 2022;(4):135 (In Russ.). https://doi.org/10.17513/spno.31790
6. Timohina OV, Hancharou AY. Characteristics of cryoprotectors used for long-term storage of donor dendritic cells. *Experimental Biology and Biotechnology.* 2021;3:102-8 (In Russ.). https://doi.org/10.33581/2521-1722-2021-3-102-108
7. Silyukova YI, Stanishevskaya OI, Pleshanov NV, Kurochkin AA. Efficiency of using a combination of mono- and disaccharides in a diluent for freezing rooster semen. *Sel'skokhozyaistvennaya biologiya*. 2020;55(6):1148–58 (In Russ.). https://doi.org/10.15389/agrobiology.2020.6.1148rus
8. Jahan S, Kaushal R, Pasha R, Pineault N. Current and Future Perspectives for the Cryopreservation of Cord Blood Stem Cells. *Transfus Med Rev.* 2021;35(2):95–102. https://doi.org/10.1016/j.tmrv.2021.01.003
9. Tas RP, Sampaio-Pinto V, Wennekes T, van Laake LW, Voets IK. From the freezer to the clinic: Antifreeze proteins in the preservation of cells, tissues, and organs. *EMBO Rep*. 2021;22(3):52162. https://doi.org/10.15252/embr.202052162
10. Whaley D, Damyar K, Witek RP, Mendoza A, Alexander M, Lakey JR. Cryopreservation: An Overview of Principles and Cell-Specific Considerations. *Cell Transplant*. 2021;30:963689721999617. https://doi.org/10.1177/0963689721999617
11. Ma J, Zhang X, Cui Z, Zhao M, Zhang L, Qi H. Investigation into antifreeze performances of natural amino acids for novel CPA development. *J Mater Chem B*. 2023;11(18):4042–9. https://doi.org/10.1039/d3tb00131h
12. Zhong Y, McGrath JK, Gong B. Dipropinonates of Sugar Alcohols as Water-Soluble, Nontoxic CPAs for DMSO-Free Cell Cryopreservation. *ACS Biomater Sci Eng*. 2021;7(10):4757–62. https://doi.org/10.1021/acsbiomaterials.1c00995
13. Kit OI, Gnennaya NV, Filippova SYu, Chembarova TV, Lysenko IB, Novikova IA, et al. Cryostorage of peripheral blood hematopoietic stem cells in transplantology: current status and prospects. *Cardiovascular Therapy and Prevention*. 2023;22(11):3691 (In Russ.). https://doi.org/10.15829/1728-8800-2023-3691
14. Dou M, Lu C, Sun Z, Rao W. Natural cryoprotectants combinations of l-proline and trehalose for red blood cells cryopreservation. *Cryobiology*. 2019;91:23–9. https://doi.org/10.1016/j.cryobiol.2019.11.002
15. Craveiro R, Castro V, Viciosa M, Dionísio M, Reis R, Duarte A, et al. Influence of natural deep eutectic systems in water thermal behavior and their applications in cryopreservation. *Journal of Molecular Liquids*. 2021;329:115533. https://doi.org/10.1016/j.molliq.2021.115533
16. Bordin DS, Indeikina LKh, Vinnitskaya EV, Danilov MA, Sabelnikova EA, Lactulose: The Advantages and Place of the Drug in Clinical Recommendations. *Effective pharmacotherapy*. 2023;19(35):42–9. (In Russ.). https://doi.org/10.33978/2307-3586-2023-19-35-42-49
17. Ait-Aissa A, Aider M. Lactulose: production and use in functional food, medical and pharmaceutical applications. Practical and critical review. *Int. J. Food Sci. Technol*. 2014;49(5):1245–53. https://doi.org/10.1111/ijfs.12465
18. Ryabtseva SA, Khramtsov AG, Budkevich RO, Anisimov GS, Chuklo AO, Shpak MA. Physiological effects, mechanisms of action and application of lactulose. *Voprosy pitaniia*. 2020;89 (2):5–20 (In Russ.). https://doi.org/10.24411/0042-8833-2020-10012
19. Killer J, Bunešová VN, Modráčková N, Vlková E, Pechar R, Šplíchal I. Lactulose in combination with soybean lecithin has a cryoprotective effect on probiotic taxa of bifidobacteria and Lactobacillaceae. *Lett Appl Microbiol*. 2023;76(2): ovad008. https://doi.org/10.1093/lambio/ovad008
20. Sidorkevich SV, Grishina GV, Remizova MI, Krasilshchikova IV, Yudina VA, Kolesov AA. Influence of negative temperatures, cryoprotectors and cryopreservatives on the structural and functional state of blood cells. *Transfusiology*. 2022;4:374–88 (In Russ.). EDN: IKXPOP
21. Dayal R, Beyls E, Vral A, Baeyens A. The Micronucleus Assay on Cryopreserved Whole *Blood. J Vis Exp*. 2024;204. https://doi.org/10.3791/65855
22. Blackwell AD, Garcia AR, Keivanfar RL, Bay S. A field method for cryopreservation of whole blood from a finger prick for later analysis with flow cytometry. *Am J Phys Anthropol*. 2021;174(4): 670-685. https://doi.org/10.1002/ajpa.24251
23. Davydkin IL, Fyodorova OI, Zaharova NO, Seleznyov AV. Computer morphometry of peripheric blood lymphocytes at patients of different age with pneumonia. *Proceedings of the Samara Scientific Center of the Russian Academy of Sciences.* 2010;12(7): 1737–41. (In Russ.).
24. Kiryanova GYu, Volkova SD, Kasyanov AD, Grishina GV, Golovanova IS, Chechetkin AV. Cryopreservation of erythrocytes at –40°C and –80°C. *Bulletin of the International Academy of Cold.* 2017;1:72–8 (In Russ.). https://doi.org/10.21047/1606-4313-2017-16-1-72-78
25. Isaeva NV, Minaeva NV, Utemov SV, Sherstnev PhS, Zorina NA, Zmeeva YuS, et al. Viability of mononuclear cells in leukocyte concentrates at the stages of their preparation, freezing, and thawing. *Bulletin of Siberian Medicine*. 2023;22(2):46–52 (In Russ.). https://doi.org/10.20538/1682-0363-2023-2-46-52
26. Vetoshkin KA, Utemov SV, Sherstnev PhS, Knyazev MG,

Kostyaev AA. Results of donors' blood platelets concentrates cryopreservation at low and ultra-low temperatures. *Transfusiology*. 2015;2(16):22–7 (In Russ.).
EDN: VVHTIX
27. Vlasov AA, Andrusenko SF, Denisova EV, Elkanova AB, Kadanova AA, Melchenko EA, et al. Morphofunctional state of cryopreserved blood cells at moderate low temperature. *Bulletin of RSMU*. 2024;(5) (In Russ.).
https://doi.org/10.24075/vrgmu.2024.038
28. Shirokikh IG, Polezhaeva TV, Shirokikh AA, Khudyakov AN, Sergushkina MI, Nazarova JI, et al. Cryoprotective Properties of the Polysaccharide Fraction of the Mushroom Hericium erinaceus BP 16. *Bulletin of the Russian Academy of Sciences. Biological Series*. 2020;(1):5–11 (In Russ.).
https://doi.org/10.31857/s0002332920010129
29. Murray A, Kilbride P, Gibson M. Trehalose in cryopreservation. Applications, mechanisms and intracellular delivery opportunities. *RSC Med. Chem.* 2024;15:2980–95.
https://doi.org/10.1039/D4MD00174E
30. Andrusenko SF, Vlasov AA, Rybchinskaya EE, Sorokina UE. Cryoprotector for whole blood. Patent of the Russian Federation № 2816446; 2024 (In Russ.).

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