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Viability of mononuclear cells in leukocyte concentrates at the stages of their preparation, freezing, and thawing

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ABSTRACT

Aim. To evaluate the viability of mononuclear cells (MNCs) in leukocyte concentrates (LCs) at the stages of their preparation, freezing, and thawing.

Materials and methods. The study material included 44 LCs from donors of allogeneic hematopoietic stem cells (HSCs) and 189 autologous LCs from patients with oncohematological disorders. LCs were obtained from donors and patients by leukocytapheresis after mobilization of HSCs. LCs from patients were frozen with dimethyl sulfoxide (DMSO) used as a cryoprotectant at a final concentration of 5% and stored in liquid nitrogen. LCs were thawed before transplantation. A total of 161 LCs were immediately transfused to the recipient after thawing, and 28 LCs were washed from DMSO before transfusion. Flow cytometry was used to determine the percentage of MNC populations that excluded 7-aminoactinomycin D (7-AAD).

Results. The viability of peripheral blood MNCs in donors and patients was close to 100%. It was found that leukocytapheresis and cryopreservation with DMSO did not affect the viability of MNCs. The freezing of LCs with DMSO, storage in liquid nitrogen, and thawing resulted in a significant decrease in the content of viable MNCs ($p = 0.0025$), while no effect of LC storage duration on the viability of MNCs was revealed. Following DMSO removal from LCs, significantly more HSCs remained in a viable state than without washing (94.4 [94.5; 95.2] % vs. 86.7 [67.6; 92.9] %, ($p = 0.0051$); for other MNC populations, except monocytes, the differences in the viability index were also statistically significant.

Conclusion. The viability of MNCs in LCs is recommended to be used as an independent characteristic of the transplant quality. In obtaining LCs and mixing them with the cryoprotectant DMSO, the viability of MNCs does not decrease, while in thawed LCs, it decreases significantly. Thawing of LCs with removal of DMSO allows to achieve the best viability of HSCs and most MNC populations.

Keywords: leukocyte concentrate, viability, 7-aminoactinomycin D, mononuclear cells, hematopoietic stem cells, dimethyl sulfoxide

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Жизнеспособность ядросодержащих клеток в лейкоконcentратах на этапах их получения, замораживания и декриоконсервирования

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РЕЗЮМЕ

Цель – оценить жизнеспособность ядросодержащих клеток (ЯСК) в лейкоконcentратах (ЛК) на этапах их получения, замораживания и декриоконсервирования.

Материалы и методы. Материал исследования – 44 ЛК доноров аллогенных гемопоэтических стволовых клеток (ГСК) и 189 аутологичных ЛК онкогематологических больных. Лейкоконcentраты доноров и больных получали методом автоматического лейкоцитафереза после мобилизации ГСК. Лейкоконcentраты больных замораживали под защитой диметилсульфоксида (ДМСО) с конечной концентрацией 5% и хранили в жидком азоте. Лейкоконcentраты декриоконсервировали перед трансплантацией, 161 ЛК после декриоконсервирования сразу переливали реципиенту, 28 ЛК перед переливанием отмывали от ДМСО. Процент не пропускающих 7-аминоактиномицин D (aminoactinomycin D, 7-AAD) популяций ЯСК определяли методом проточной цитофлуориметрии.

Результаты. Жизнеспособность ЯСК периферической крови доноров и больных приближалась к 100%. Показано отсутствие влияния аппаратного лейкоцитафереза и процедуры смешивания с ДМСО на жизнеспособность ЯСК. Замораживание ЛК под защитой ДМСО, хранение в жидком азоте и их декриоконсервирование приводили к значимому снижению содержания жизнеспособных ЯСК ($p = 0,0025$), при этом влияние длительности хранения ЛК на жизнеспособность ЯСК не выявлено. В результате отмывания от ДМСО в жизнеспособном состоянии сохраняется существенно больше ГСК, чем без отмывания (94,4 [94,5; 95,2]% против 86,7 [67,6; 92,9]%; $p = 0,0051$); для других популяций ЯСК, кроме моноцитов, различия показателя жизнеспособности также статистически значимы.

Заключение. Жизнеспособность ЯСК в ЛК рекомендуется использовать как самостоятельную характеристику качества трансплантата. В процессе получения ЛК и их смешивания с криоконсервантом ДМСО жизнеспособность ЯСК не снижается, а в декриоконсервированных ЛК значительно падает. Декриоконсервирование ЛК с отмыванием от ДМСО позволяет достигать лучшей жизнеспособности ГСК и большинства популяций ЯСК.

Ключевые слова: лейкоконcentрат, жизнеспособность, 7-аминоактиномицин D, ядросодержащие клетки, гемопоэтические стволовые клетки, диметилсульфоксид.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is an effective method of therapy for hemoblastosis and hematopoietic depressions [1, 2]. In modern conditions, the transplant material, leukocyte concentrates

(LCs) with a sufficient content of hematopoietic stem cells (HSCs), can be obtained by leukocytapheresis. If it is necessary to preserve the transplant material for a long time (more than 72 hours), the technologies of its freezing with cryoprotectants are used. [3]. Kirov Re-

search Institute of Hematology and Blood Transfusion of the Federal Medical and Biological Agency has accumulated many years of experience in carrying out transplantations of autologous and allogeneic HSCs and harvesting allogeneic HSCs for other transplant centers [4].

Obtaining transplant material involves several technological procedures that ensure stable transplant function and guarantee the success of HSCT. The ability to restore hematopoiesis in the recipient is directly related to the transfused dose of HSCs. In accordance with the requirements of the European Society on Blood and Bone Marrow Transplantation, quality control of the cellular product is mandatory, the calculation of HSC is required, and it is necessary to assess cellular elements that are potentially capable of harming the patient. In cases where the product is subject to any manipulations affecting its composition, confirmation of the viability of cells in the product becomes particularly relevant (URL: <https://www.ebmt.org/8th-edition-fact-jacie-standards>, accessed: 13.10.21).

Currently, HSCs are evaluated in accordance with the recommendations of the International Society of Hematotherapy and Graft Engineering (ISHAGE) [5]. A HSC-containing LC is a sufficiently heterogeneous cellular product [6]. The ISHAGE protocol regulating the calculation of HSCs is widely used for the certification of a cell product, but such an assessment does not provide any information about the viability of cells. Viable MNCs are cells whose membrane remains non-permeable to nuclear staining dyes. Viability testing can be based on supravital staining of cell samples with various dyes. Currently, it has become possible to use fluorescent DNA dyes, such as propidium iodide, 7-aminoactinomycin D, and SYTO dyes [7].

A decrease in the number of viable leukocytes during LC storage can negatively affect the calculated value of the target transplantation dose of HSCs and the parameters of engraftment [8], while an increase in the proportion of non-viable cells in LC can lead to the emergence of various cell degradation products in it [9].

The aim of the study was to evaluate the viability of MNCs in LCs at the stages of their preparation, freezing, and thawing.

MATERIALS AND METHODS

The material for the study was blood and LC samples from donors of allogeneic HSCs, as well as patients with oncohematological disorders. The blood samples were taken for examination immediately before the start of leukocytapheresis. The LCs of the donors and patients were harvested using an anticoagulant for automatic leukocytapheresis. The LCs of the patients were cryopreserved by mixing with a solution of DMSO at a final concentration of 5% and dextran. LCs were frozen in liquid nitrogen vapors at 30–35 cm above the surface of liquid nitrogen (–145...–160 °C), followed by transfer to the liquid nitrogen medium.

Immediately before transplantation, 161 LCs were thawed in an aqueous medium at a temperature of 39–41 °C, then the LCs were transfused to the patient. Another part of HSC-containing LCs ($n = 28$) was washed from DMSO after thawing by adding a mixture of albumin and polyglukin to the cell suspension (in a ratio of 1:4). After centrifugation at 2,000g for 5 min, the supernatant was removed, and the cell precipitate was resuspended in a mixture of albumin and polyglukin. The stages of preparation of the transplant material are shown in Fig. 1.

Stages of studying the viability of MNCs	Transplantation material	
	Allogenic HSCs ($n = 44$)	Allogenic HSCs ($n = 44$)
1. Mobilization of HSCs	peripheral blood	peripheral blood
2. Obtaining LCs by leukocytapheresis	native LCs	native LCs
3. Mixing LCs with DMSO		LCs mixed with DMSO
4. Freezing LCs, storage of LCs in liquid nitrogen vapor, thawing of LCs	LCs without washing from DMSO ($n = 161$)	LCs washed from DMSO ($n = 28$)

Fig. 1. Assessment of MNC viability (stages, material)

Table 1

Content of viable mononuclear cells in leukocyte concentrates, <i>Me</i> (Q_{25} ; Q_{75})		
Stages of viability control of MNCs in LCs		Content of 7-AAD- negative cells, %
Native LC, $n = 189$	I	98.4 (97.5; 99.8)
LC mixed with DMSO, $n = 189$	II	96.1 (94.0; 98.8)
LC without washing from DMSO, $n = 161$	III	80.6 (76.0; 89.4)
LC after washing from DMSO, $n = 28$	IV	92.2 (85.2; 96.4)
p		I–II = 0.1124; II–III = 0.0025; II–IV = 0.0087; III–IV = 0.0054

Blood and LC samples were diluted with a phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin to achieve a leukocyte concentration of $5 \times 10^9/l$. The samples were incubated in the dark for 15 min at a temperature of 20–24 °C with conjugates of monoclonal antibodies to CD45 and CD34 with fluorochromes, fluorescein isothiocyanate, and phycoerythrin, respectively, and with a 7-AAD solution. The samples were tested on the BD FACSCanto™ II flow cytometer (BD Biosciences, USA). We took into account the proportion of 7-AAD-negative events in each MNC population.

STADIA (Russian Federation) and Microsoft Excel were used for statistical analysis of the obtained results. The data were presented as the median and the interquartile range *Me* (Q_{25} ; Q_{75}). We used the Van der Waerden test to perform a comparative analysis of unrelated aggregates and the Wilcoxon test for related ones. In multiple comparisons, the values of the criteria were determined using the Bonferroni correction. The correlation was established by calculating the Spearman's rank correlation coefficient. The differences were considered statistically significant at $p < 0.05$.

RESULTS

The number of 7-AAD-negative MNCs in the blood taken before the start of leukocytapheresis from the donors and patients turned out to be comparable (99.8 (97.5; 99.9)% and 99.7(98.1; 99.9)%, respectively, $p = 0.1109$). The content of viable MNCs in native LC in the donors was 99.6 (98.5; 99.8)%, in the patients – 98.4 (97.5; 99.8)%. The obtained values did not differ from those in the blood samples from which LCs were prepared ($p = 0.1241$ for the donor group; $p = 0.0893$ for the patient group).

The difference in the proportion of 7-AAD-negative MNCs in native LCs of the patients and in LCs mixed with DMSO was not statistically significant (Table 1). There was a significant decrease in the viability of MNCs in LCs after thawing. An increase in the permeability of the MNC membranes for the vital 7-AAD dye was recorded in the LC samples that were thawed both without additional manipulation – washing from DMSO, and with it. The relative number of 7-AAD-negative MNCs in the LCs that were not washed from DMSO turned out to be significantly lower than in the LCs after washing from DMSO.

The relationship between the proportion of viable cells and the storage time of patients' LCs in the frozen state before thawing and transplantation was studied, a correlation between these two parameters was not revealed ($r = 0.11$).

Fig. 2 shows the distribution of thawed LCs of the patients according to the content of viable MNCs in them. Three ranges of viability were taken into account: 80% or less, 90–80.1%, and 95.5–90.1%. In the LCs washed from DMSO, critical levels (from 80% to 62.2%) of MNC viability were recorded in 7.1% (2 out of 28) of cases, in the unwashed LCs – in 44.1% (71 out of 161) of cases.

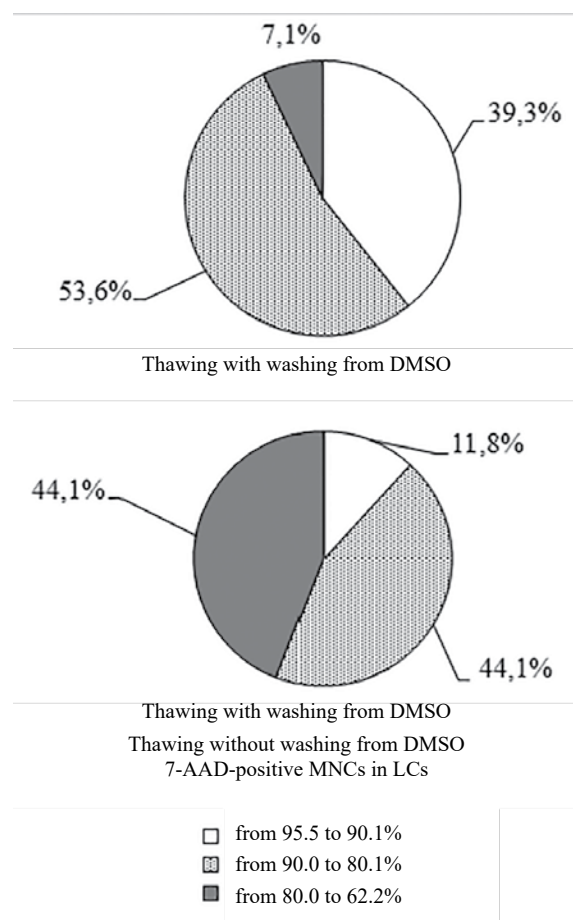


Fig. 2. Distribution of thawed leukocyte concentrates according to the content of viable mononuclear cells

Among all MNCs, the HSC content was 0.6%; lymphocytes – 44.6%, monocytes – 31.5%, granulocytes – 21.9%, erythrokaryocytes – 1.4%. The population of granulocytes that were thawed and were not washed from DMSO turned out to be the least viable

(Table 2). In the LCs thawed with subsequent washing from DMSO, higher viability values of most cell populations were revealed compared to those in the LCs which were not washed. The only exception was the population of monocytes.

Table 2

Content of viable mononuclear cells in thawed leukocyte concentrates, $Me(Q_{25}; Q_{75})$			
Types of MNCs	The content of 7-AAD-negative cells		<i>p</i>
	in LCs without washing from DMSO, %	in LCs washed from DMSO, %	
Leucocytes	81.8 (75.1; 90.4)	89.3 (86.3; 93.7)	0.0338
HSC	86.7 (67.6; 92.9)	94.4 (94.5; 95.2)	0.0051
– lymphocytes	93.4 (87.9; 96.9)	95.5 (93.5; 97.7)	0.0455
– monocytes	94.9 (93.5; 96.0)	97.4 (98.1; 92.6)	0.0718
– granulocytes	36.7 (22.8; 53.5)	58.8 (52.1; 65.6)	0.0006
Erythrokaryocytes	69.0 (56.6; 82.9)	90.8 (84.9; 95.5)	0.0008

DISCUSSION

Supravital cell staining with an automatic analysis of the results in laser flow cytofluorometry was used to assess the viability of MNCs in LCs. The 7-AAD dye is easily embedded between cytosine and guanine bases and is detected in the red region of the visible spectrum (635–675 nm). If simultaneous staining of cells with fluorochrome-conjugated monoclonal antibodies to cellular determinants is necessary, 7-AAD is considered preferable among fluorescent DNA dyes [10]. The percentage of 7-AAD-negative MNCs should be recommended as an informative parameter of LC control at technological stages of production and storage.

As a result of the conducted research, it is shown that the viability of blood and native LCs is approaching 100%, the variation of the parameter is extremely insignificant. The information obtained is consistent with the results of the study on stem cell viability, in which several DNA dyes were used [7]. The number of 7-AAD-negative MNCs in HSC-containing LCs and the blood from which they were obtained coincided. Consequently, leukocytapheresis and anticoagulant addition do not have a negative effect on the permeability of MNC membranes.

Since 2003, highly purified DMSO at a concentration of 10% has been used for cryopreservation of transplant material. The molecular basis of the DMSO effect on cell membranes is still being investigated [11]. It is known that various concentrations of DMSO have fundamentally different mechanisms of action on HSCs [12]. The information given in the literature on the viability of cryopreserved HSCs, their repopulating ability, and the timing of hematopoiesis recovery

[13, 14, 3] do not allow to draw final conclusions about the optimal DMSO concentration.

A possible effect of concentrated DMSO at the stage of its introduction into the LCs was studied. The number of 7-AAD-negative MNCs in LCs mixed with DMSO at a final concentration of 5% and in native LCs did not differ significantly. The conclusion was made about the reasonability of the method used for introducing a concentrated DMSO solution into the cell product. A significant decrease in the MNC viability in thawed LCs was revealed both with and without washing from DMSO, compared with that in native LCs mixed with a cryoprotectant. The results obtained are consistent with the literature data [15]. The relationship between the storage time (from 7 to 120 days) and the number of 7-AAD-negative MNCs released from cold suspended animation was not confirmed, freezing and thawing are probably the most critical for the permeability of MNC plasma membranes.

It is known that DMSO can have a toxic effect on the human body when used as a cryoprotectant [16]. To reduce the toxic effect of DMSO on the recipient's body, such approaches as lowering the hematocrit of the cell product [17], washing HSC-containing cell products [18], and using ice recrystallization inhibitors [8] are studied. Non-viable MNCs may have a fragmented membrane or represent naked nuclei [9]. It was found, that in the LCs washed from DMSO, there are more viable MNCs than in the LCs that were not washed. As a result of washing, the DMSO-containing resuspension solution in LCs is partially replaced with a mixture of albumin and polyglukin, the contact time of MNCs with extracellular DMSO is reduced [18].

LC is a product that is only slightly enriched with stem cells compared to peripheral blood [7]. The per-

centage of HSCs in LCs averaged 0.6%, the largest proportion among all MNCs was represented by cellular elements that do not have repopulating properties. At the same time, the number of CD45-positive cells directly affects the estimated transplantation dose of HSCs [19]. When LCs are thawed without washing, granulocytes undergo the greatest destruction, which is consistent with the reports of researchers [20]. Given high sensitivity of membranes of granulocytic components and their significant proportion in LCs, the granulocyte population makes the greatest contribution to reducing the viability of all MNCs after thawing. When washing from DMSO, greater viability of most of the isolated MNC populations was observed, except for monocytes. Presumably, washing hinders the increase in the permeability of MNC plasma membranes. In addition, it cannot be ruled out that non-viable MNCs are selectively removed during LC washing.

The possibility of loading the transplant material with cell degradation products is another aspect of the negative impact that the destruction of MNC membranes has on the recipient's body. The danger of enriching blood components with such substances during storage was discussed [9].

CONCLUSION

The viability of MNCs in LCs is recommended to be used as an independent characteristic of the transplant quality. In the process of obtaining LCs and mixing them with DMSO, the viability of MNCs does not decrease, while in thawed LCs, it decreases significantly. Thawing of LCs with the removal of DMSO allows to achieve the best viability of HSCs and most MNC populations.

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Authors' contribution

Isaeva N.V. – conception and design, analysis and interpretation of data, drafting of the manuscript. Minaeva N.V. – critical revision of the manuscript for important intellectual content. Sherstnev Ph.S., Utemov S.V., Zmeeva Yu. S., Butolina M.A. – collection, analysis and interpretation of data. Zorina Natalia A. – selection and management of donors and patients.

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