either at high concentrations or inadequate removal before transplantation [7, 12]. This fact determines the relevance of research aimed at optimizing DMSO concentration and cryopreservation conditions.

Notably, during cryopreservation, cells are exposed to oxidative stress [11], accompanied by accumulation of reactive oxygen species (ROS) and damage to cell membranes, proteins, and DNA. To minimize these negative effects, antioxidants are increasingly used, including Trolox, a synthetic analogue of vitamin E [8, 14, 20]. The ability to reduce ROS levels and maintain cell viability makes it promising for use in cryoprotective media.

Based on the above, the aim of the research was to determine the quantitative and qualitative characteristics of human cord blood nucleated cells, including hematopoietic progenitor cells, after cryopreservation in solutions with different concentrations of dimethyl sulfoxide and a water-soluble analogue of vitamin E: antioxidant Trolox, and after *in vitro* transfusion simulation.

MATERIALS AND METHODS

In this study, we used the human cord blood, procured from the umbilical vein following a normal delivery with the informed consent of laboring woman, in accordance with the requirements of the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine (Kharkiv). Blood was collected in a 250 ml glass bottle containing glucose-citrate anticoagulant (Biopharma, Ukraine). Nucleated cells from whole CB were isolated by sedimentation in a 6% solution of polyglucin with a molecular weight of 60,000 [2]. For this purpose, polyglucin (Yuria-Pharm, Ukraine) was added to the blood bottle in a ratio of 1 : 1, mixed thoroughly and left for 30—50 min until a clear boundary was formed between the erythrocyte layer and the supernatant. The supernatant was collected in a separate 100 ml glass vial and centrifuged for 15 min at 800g to precipitate the cell fraction and concentrate it further.

The solution of antioxidant Trolox (Sigma-Aldrich, USA) was prepared using DMSO as a basis, and added to the experimental samples to final concentrations of 20; 30; 50; 70 and 200 μM. The experimental samples were supplemented with appropriate concentrations of antioxidant and a 25% DMSO solution (Starlab, USA), prepared in a 6% polyglucine solution, to final concentrations in the sample of 2.5, 5, and 7.5%. The samples were treated with DMSO dropwise under constant stirring in an ice bath at 0—4 °C. The equilibration time with cryoprotectant before transferring samples to freezer was 20 min.

Sample were frozen in Nunc cryotubes (Thermo Fisher Scientific, USA) at a rate of 1 °C/min down to –80 °C using a programmable freezer (Special Designing and Technological Bureau at the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine) with subsequent immersion in liquid nitrogen [2]. Samples were thawed at 37–40 °C in a water bath with a constant shaking until the solid phase disappeared.

The *in vitro* transfusion was simulated by transferring a portion of thawed NC suspension to Hanks' solution (1 : 10 by volume, pH 7.4) with incubation at 37 °C [18] for 1 hr. This was followed by centrifugation at 800g for 5 min and subsequent removal of the supernatant.

The absolute number of cord blood NCs in the samples was calculated in a Goryaev chamber according to the standard method [5].

Cell losses during freeze-thawing and *in vitro* transfusion simulation (preservation of NCs) were calculated using the formulae:

$$\text{Survival of NCs (HPCs) after cryopreservation} = \frac{\text{Number of cells after cryopreservation}}{\text{Initial number of cells before cryopreservation}} \times 100\ \%,$$

$$\text{Survival of NCs (HPCs) after transfusion} = \frac{\text{Number of cells after } \textit{in vitro} \text{ transfusion simulation}}{\text{Initial number of cells before cryopreservation}} \times 100\ \%,$$

The viability of NCs and HPCs was assessed using the standard ISHAGE protocol (International Society of Hematotherapy and Graft Engineering) with monoclonal antibodies CD45 FITC and DNA dye 7-aminoactinomycin D (7-AAD) by flow cytometry [13]. For this purpose, 10 μl of reagents (FITC-labeled CD45, clone 2D1, PE-labeled CD34, clone 8G12 and vital DNA dye 7AAD) were added to 50 μl of cell suspension, mixed and incubated for 15 min at room temperature in the dark. To destroy erythrocytes, 1 ml of lysing solution "BD Pharm

Lyse™ Lysing Buffer" (BD, USA) was added to each tube. The samples were analyzed using the "CELLQuest Pro" (BD) software for flow cytometry. To minimize the error, 20,000 events (ECV) or at least 200 $CD34^+$-cells were analyzed.

The yield of viable nucleated ($CD45^+7AAD^-$) and hematopoietic progenitor ($CD34^+7AAD^-$) cells after cryopreservation (*in vitro* transfusion simulation) compared to their initial level before cryopreservation was determined by the formulae:

$$\text{Yield of viable cells after cryopreservation} = \frac{\text{Absolute number of viable cells after cryopreservation}}{\text{Absolute number of viable cells before cryopreservation}} \times 100\ \%,$$

$$\text{Yield of viable cells after } \textit{in vitro} \text{ transfusion simulation} = \frac{\text{Absolute number of viable cells after } \textit{in vitro} \text{ transfusion simulation}}{\text{Absolute number of viable cells before cryopreservation}} \times 100\ \%,$$

The absolute number of viable cells prior to cryopreservation was equal to the product of the absolute number of cells preserved in the sample before cryopreservation and the percentage of viable cells in the sample before cryopreservation (determined by flow cytometry as $7AAD^-$-cells).

The absolute number of viable cells after cryopreservation (after *in vitro* transfusion simulation) was equal to the product of the absolute number of cells preserved in the sample after cryopreservation (after *in vitro* transfusion simulation) and the percentage of viable cells in the sample after cryopreservation (after *in vitro* transfusion simulation) (determined by flow cytometry as $7AAD^-$-cells).

Results were statistically processed using the "Statgraphics plus 2.1." software package (Manugistics Corp., USA). The data are presented as M ± SE (arithmetic mean ± standard error). The statistical significance of the results was determined by the nonparametric Mann-Whitney method at $p < 0.05$. The number of experiments in each series of experiments was at least five.

RESULTS AND DISCUSSION

Our previous studies [21] have shown that during cryopreservation, the cord blood NCs undergo significant stress, resulting in accumulation of high concentrations of ROS. According to P.M. Henson *et al.* [9], these molecules can initiate the development of apoptosis and cell death before and especially after cryopreservation. There is also a theory that during cryopreservation, cells receive certain damage or signals that make them "prone" to the development of apoptotic/necrotic processes, but do not initiate their development at this point [3]. In addition, it is known that during cryopreservation, the activity of antioxidant system decreases, the order of membrane structure is disrupted, and glutathione is released from organelles, which can increase the ROS level in cells after their transfer to the bloodstream with the development of lipid peroxidation (LPO). Also, N.S. Pushkar *et al.* [15] suggested that the maximum development of LPO, for example, in thawed mitochondria, was not detected immediately after warming, but after a certain time interval, usually several hours. There is an assumption that immediately after warming, the LPO level is limited by antioxidants of glutathione peroxidase system [16]. As the natural defense mechanisms (antioxidants) are destroyed/depleted after warming in mitochondria, the oxidation processes increase. One of the specific manifestations of lipid oxidation is the rapid development of LPO in the membrane, when any ultrastructural disorders are not detected. This specific feature of LPO development in mitochondrial membranes after low temperature exposure and during storage is described in detail by the well-known cryobiological phenomenon: cold damage latency. In view of the above, first of all, to predict the preservation of structural and functional properties of human cord blood NCs, as well as their therapeutic efficacy, it is important not only to assess the number of cells immediately after thawing, but also to determine the delayed survival, which we modeled by transferring cell aliquot to physiological conditions *in vitro*.

For this purpose, we used here a simple model, reproducing only the basic principles of transfusion: dilution of frozen-thawed cell suspension, which occurs naturally in the recipient's bloodstream, maintaining the iso-osmoticity of the medium, and incubation temperature of 37 °C [18]. Maintaining the temperature of 37 °C throughout the incubation period is an important factor in detecting metabolic disorders, since the cell survival at lower temperatures masks a possible imbalance in metabolism due to the slowdown of all biochemical processes. Thus, this *in vitro* model of physio-

logical conditions provides precise control of the main environmental parameters necessary for detecting damage to cryopreserved cord blood NCs, determining their stability depending on the approach used for cryopreservation. In addition, such a relatively simple methodological approach should promote the development of safe and effective biomedical research and optimize transfusion therapy.

At the first stage of research, it was expedient to evaluate the changes in quantitative and qualitative characteristics of cord blood NCs cryopreserved with different concentrations of DMSO after 1-hour incubation under physiological conditions *in vitro* (control samples) compared to similar samples analyzed immediately after thawing.

The analysis of the results of cord blood NCs survival, including HPCs, showed this index to be minimal in the samples cryopreserved with DMSO at a concentration of 2.5% (Table 1).

In the mentioned experimental groups, the loss of the absolute number of cells was significantly higher (up to twice) than immediately after thawing. This is primarily due to the insufficient DMSO concentration for cryopreservation. In the samples containing 5% DMSO, a 30—40% decrease in the survival rate was observed compared to the data after thawing. Cells cryopreserved with DMSO at a concentration of 7.5% proved to be the most protected (Table 1). At this concentration, a decrease in survival rates was less pronounced compared to other experimental groups (2.5 and 5% DMSO), although it was significantly lower by 25—35% compared to the data immediately after thawing.

It should be noted that such an index as cell viability ($7AAD^-$-cells) is relative, since in each sample analyzed with a flow cytometer, any number of cells remaining in the sample is taken as 100%. However, this analysis does not take into account quantitative indices, in particular, the absolute number of cells (cell loss/destruction). The Table 1 shows that due to significant destruction during cryopreservation, primarily of dead or damaged cells, the number of cord blood $7AAD^-$-NCs after 1-hour incubation under conditions that simulate physiological ones significantly exceeds the number of viable cells determined right after thawing. Based on this, in order to objectively assess the effectiveness of cryopreservation of the studied samples, we determined the number of viable $CD45^+$-cells in the samples compared to their initial number before cryopreservation (the yield of viable cells) in addition to controlling the quantitative and qualitative indices of HPC population, which are the main effectors in therapy of various diseases.

These findings showed that transferring the frozen-thawed cells to physiological conditions *in vitro* reduced the yield of viable NCs (Fig. 1).

DMSO at concentration of 2.5% was ineffective for cryopreservation, as evidenced by the lowest yield of viable cells (up to 25—28%) compared to their initial number prior to freezing. When using 5% DMSO, the yield of viable cells after transfusion simulation averaged 35%. The highest values of this index after 1-hour incubation in Hanks' solution (up to 47%) were recorded after using a 7.5% concentration of cryoprotectant (Fig. 1).

Table 1. **Survival and viability of cord blood $CD45^+$- and $CD34^+$-cells cryopreserved in DMSO solutions of different concentrations after 1-hour incubation under physiological conditions *in vitro*, %; M ± SE**

DMSO concentration, %	Nucleated cells ($CD45^+$)		Hematopoietic progenitor cells ($CD34^+$)	
	Survival	Viability	Survival	Viability
2.5				
A	50.6 ± 4.9	50.9 ± 2.4	50.6 ± 3.2	50.4 ± 3.8
B	28.7 ± 5.0 *	76.0 ± 3.4 *	25.9 ± 2.5 *	59.7 ± 6.8
5				
A	65.2 ± 3.6	59.5 ± 3.3	73.9 ± 3.2	61.9 ± 3.2
B	38.0 ± 4.6 *	75.4 ± 2.7 *	51.2 ± 2.5 *	63.3 ± 3.6
7.5				
A	78.4 ± 3.0	67.9 ± 1.7	81.4 ± 2.9	68.9 ± 2.2
B	49.2 ± 5.0 *	74.3 ± 0.9 *	59.6 ± 9.7 *	66.7 ± 2.7

Notes: A — data obtained immediately after thawing; B — data obtained after cryopreservation and 1-hour incubation under physiological conditions *in vitro*; * significant difference compared to the corresponding control values obtained immediately after thawing; $p < 0.05$.

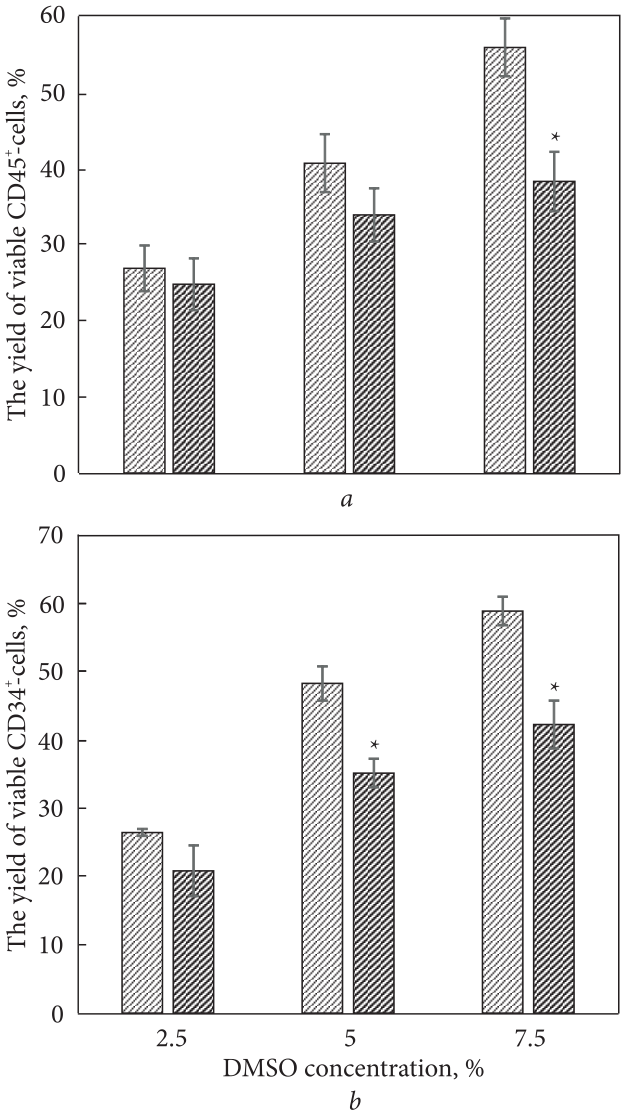

Fig. 1. Yield of viable $CD45^+$-cells (*a*) and $CD34^+$-cells (*b*) after cryopreservation in DMSO solutions of different concentrations (▨) and transfer to physiological conditions *in vitro* (▩); * — significant difference compared to the corresponding group of cells immediately after thawing, $p < 0.05$; M ± SE

At the next stage of work, to clarify the degree of involvement of oxidative processes during CB NCs cryopreservation, the number of cells with excessive ROS content was assessed. For these studies, we used a cytofluorimetric method with dichlorofluorescein diacetate (DCFH2-DA), which, after cleavage by intracellular form of diacyl esterase, loses its ability to freely leave the cell, and the formed non-fluorescent DCFH2 (in the presence of ROS) is converted into a highly fluorescent form of DCF, the fluorescence of which is directly proportional to the amount of intracellular ROS, primarily hydrogen peroxide [17].

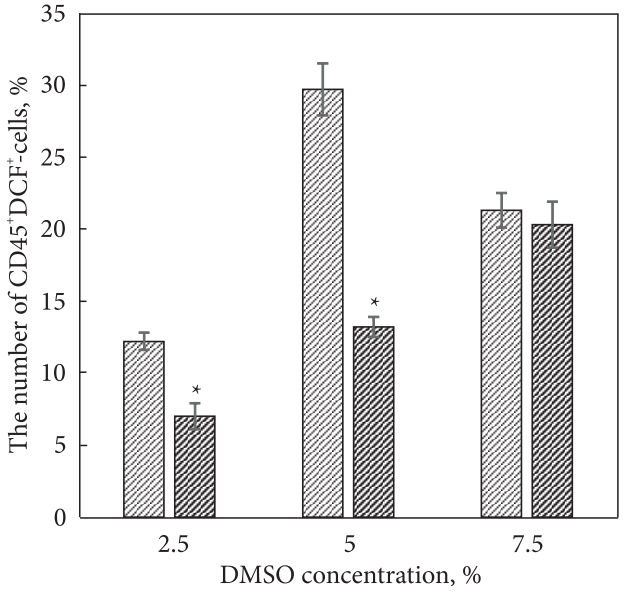

Fig. 2. Number of cord blood nucleated cells with excess ROS after cryopreservation in DMSO solutions of different concentrations (▨) and transfer to physiological conditions *in vitro* (▩); * — difference is significant compared to the corresponding group of cells immediately after thawing, $p < 0.05$; M ± SE

The analysis of the number of cells with excessive ROS content in the samples cryopreserved with ultra-low (2.5%) and low (5%) concentrations of cryoprotectant (Fig. 2) showed a significant decrease in the number of $CD45^+DCF^+$-cells by approximately 2 times compared to the data obtained immediately after thawing.

As mentioned earlier, these data may be explained by destruction of a significant number of damaged cells during a 1-hour incubation under physiological conditions *in vitro*, which correlates with the survival data (Table 1). With increasing DMSO concentration in the samples, the number of cells with high ROS content augments. This is due to the enhanced cryoprotective effect of DMSO solution, which ensures the survival of a larger number of cells in the sample containing 7.5% cryoprotectant. The high percentage of $CD45^+$ DCF^+-cells in the samples with 5% DMSO immediately after cryopreservation is probably due to the fact that this concentration of DMSO can provide high survival, but not viability of cells, and these destabilized cells are destroyed during incubation in Hanks' solution (Fig. 2, Table 1).

Thus, the found significant number of cells with excessive ROS levels in the samples after *in vitro* transfusion simulation and the resulting probable development of oxidative processes made it logical

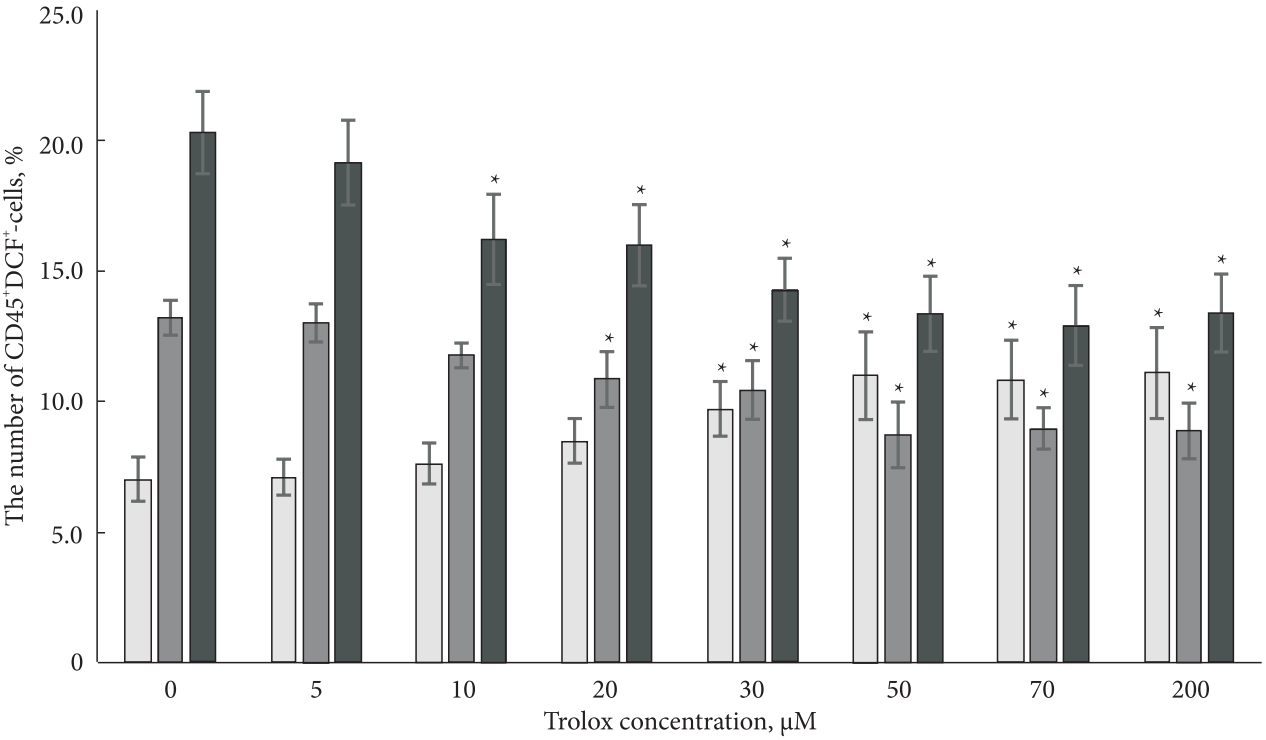

Fig. 3. Number of cord blood nucleated cells with excess ROS after cryopreservation in solutions containing DMSO and Trolox of different concentrations and *in vitro* transfusion simulation: ▫ — cryoprotective medium with 2.5% DMSO; ▪ — cryoprotective medium with 5% DMSO; ■ — cryoprotective medium with 7.5% DMSO; * — difference is significant compared to the corresponding control group without Trolox addition, $p < 0.05$; M ± SE

and consistent to proceed to the next stage of the research, namely evaluating the effectiveness of adding antioxidants to cryoprotective solutions in order to mitigate oxidative processes in cells and, as a result, increase the level of cell survival and viability after cryopreservation and 1-hour incubation under physiological conditions *in vitro*. As part of this study, the Trolox antioxidant and cytoprotective activities were determined.

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid ($C_{14}H_{18}O_4$)) is a water-soluble synthetic form of vitamin E, which is a tocopherol derivative. Trolox acts as a potent electron donor, neutralizing free radicals (superoxide, hydroxyl radical, peroxyl radicals), which helps prevent oxidative damage to biomolecules [14, 20]. After electron donation, Trolox is converted to a stable Trolox radical, being less likely to participate in oxidative chain reactions. Trolox has also been shown to effectively inhibit the lipid peroxidation, by protecting cell membranes and low-density lipoproteins from oxidation [6, 8].

Based on the above, in the next series of experiments, we determined the impact of Trolox-containing cryoprotectant solutions on the state of cord blood NCs, including HPCs, after thawing and *in vitro* transfusion simulation.

At the first stage, determining the number of nucleated cells with excessive ROS content ($CD45^+$ DCF^+) after 1-hour incubation in Hanks' solution is one of the key integral parameters for assessing the NC state both during exposure to different concentrations of cryoprotectant and after introduction of an antioxidant. The dependence of this index on DMSO concentration used for cell freezing was revealed (Fig. 3).

When using 2.5% DMSO, the number of DCF-positive cells in Trolox-containing samples increases compared to the control. At antioxidant concentrations of 30 μM and above, the differences are significant. The maximum number of ROS is recorded in the samples containing 50—200 μM Trolox. This increase in $CD45^+DCF^+$-cells cannot be considered as an unambiguously negative phenomenon, since, as will be demonstrated below, in these samples, despite the ultra-low concentration of DMSO, there is a slight increase in the number of preserved cells, although they may also be destabilized. In the samples containing 5% DMSO (Fig. 3), an opposite trend is observed: with increasing Trolox concentration in the samples, the number of cells with excessive ROS content decreases compared to the antioxidant-free control (the mini-

mum number of ROS was in samples with 50—200 μM Trolox). In the samples cryopreserved with 7.5% DMSO, similar changes occur: the number of ROS significantly reduces already at 10 μM Trolox. The maximum effect is manifested in the samples with 50–70 μM antioxidant (36.5% decrease as compared to control values) (Fig. 3).

The analysis of cord blood NC survival after cryopreservation in solutions with different concentrations of DMSO and Trolox and *in vitro* transfusion simulation revealed a pronounced significant dependence of this index on the applied concentration of cryoprotectant (Table 2). It was found that the minimum survival rates (about 29–35%) were expected in the samples containing 2.5% DMSO, and the maximum ones (up to 65%) were in those with 7.5% DMSO. When using 5% DMSO, the survival rate in the samples containing antioxidant was up to 49%.

The study of the impact of different Trolox concentrations showed no significant differences from the control in the samples containing 2.5 and 5% DMSO. In these samples, there was only a tendency towards an increase in the number of preserved cells.

The samples cryopreserved with 7.5% DMSO and Trolox at a concentration between 5—30 μM also showed a tendency to increased survival, while a significant augmentation was in the samples with 50—200 μM of antioxidant (survival increased from 21 to 28%) (Table 2).

The analysis of HPC survival showed that in the samples with 2.5% DMSO, the cell survival was minimal and amounted to about 25% in the control and up to 37% in the samples with antioxidant (Table 2). Despite the ultra-low concentration of cryoprotectant, the Trolox supplement contributed to an increase in cell survival rate (up to 38%) under these experimental conditions, while significant differences were observed in the samples containing 50—200 μM of Trolox. Increasing the cryoprotectant concentration in the freezing medium up to 5% resulted in a nearly two-fold improvement

Table 2. **Survival and viability of cord blood CD45$^+$- and CD34$^+$-cells after cryopreservation in solutions containing DMSO and Trolox of different concentrations and *in vitro* transfusion simulation, %; M ± SE**

DMSO concentration, %	Trolox concentration, μM	Nucleated cells (CD45$^+$)		Hematopoietic progenitor cells (CD34$^+$)	
		Survival	Viability	Survival	Viability
2.5	Control	28.7 ± 5.0	76.0 ± 3.4	25.9 ± 2.5	59.7 ± 6.8
	5	29.5 ± 5.1	77.1 ± 2.3	25.9 ± 2.5	60.3 ± 6.6
	10	29.5 ± 5.1	76.8 ± 2.8	25.9 ± 2.5	60.4 ± 6.7
	20	30.8 ± 5.1	77.0 ± 2.8	30.0 ± 3.0	57.4 ± 6.9
	30	31.9 ± 4.7	76.4 ± 2.0	31.4 ± 2.3	58.3 ± 7.0
	50	341 ± 4.1	76.5 ± 3.2	35.3 ± 1.8 *	56.4 ± 6.5
	70	33.8 ± 4.0	76.7 ± 2.7	35.7 ± 2.0 *	56.0 ± 6.3
	200	33.5 ± 4.4	76.5 ± 2.9	36.1 ± 1.2 *	54.7 ± 6.8
5	Control	38.0 ± 4.6	75.4 ± 2.7	51.2 ± 2.5	63.4 ± 3.6
	5	39.5 ± 4.6	76.1 ± 2.7	51.9 ± 2.4	64.2 ± 3.7
	10	39.5 ± 4.6	76.6 ± 2.4	51.9 ± 2.4	64.8 ± 3.7
	20	40.1 ± 4.8	76.7 ± 1.6	56.4 ± 1.2 *	63.1 ± 2.9
	30	41.5 ± 4.7	77.2 ± 1.3	61.5 ± 2.8 *	61.8 ± 3.4
	50	44.5 ± 4.2	76.1 ± 1.9	62.6 ± 2.4 *	62.0 ± 2.6
	70	44.8 ± 4.0	75.7 ± 2.0	62.6 ± 2.4 *	62.1 ± 3.1
	200	44.5 ± 4.0	75.2 ± 1.9	60.4 ± 3.0 *	61.4 ± 2.5
7.5	Control	49.2 ± 4.3	74.3 ± 0.9	59.6 ± 4.8	66.7 ± 2.7
	5	50.0 ± 5.0	74.4 ± 0.6	59.6 ± 4.8	67.2 ± 2.7
	10	50.0 ± 5.0	75.2 ± 0.8	59.6 ± 4.8	68.0 ± 2.8
	20	52.8 ± 4.6	74.5 ± 0.4	65.0 ± 4.9	66.0 ± 3.1
	30	53.9 ± 4.8	75.6 ± 1.0	66.6 ± 5.3	65.2 ± 3.0
	50	59.3 ± 4.1 *	75.6 ± 0.3	71.1 ± 3.5 *	63.7 ± 3.2
	70	59.6 ± 3.9 *	74.9 ± 0.5	71.5 ± 3.1 *	64.1 ± 3.5
	200	59.6 ± 4.0 *	73.1 ± 0.6	70.5 ± 3.7 *	62.5 ± 3.2

Note: * the difference is significant compared to the corresponding control group without Trolox addition, $p < 0.05$.

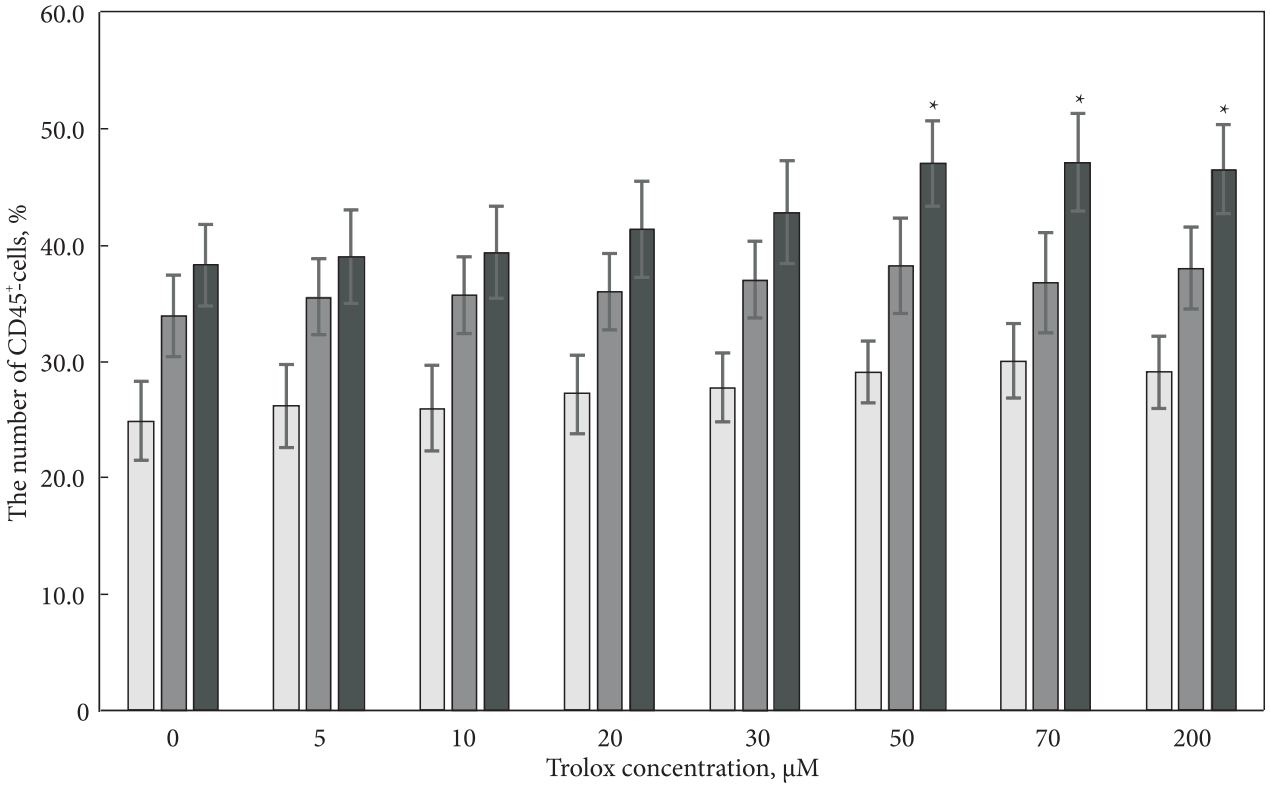

Fig. 4. Yield of viable $CD45^+$-cells after cryopreservation in solutions containing DMSO and Trolox of different concentrations and *in vitro* transfusion simulation compared to their initial level before freezing: ▫ — cryoprotective medium with 2.5% DMSO; ▪ — cryoprotective medium with 5% DMSO; ■ — cryoprotective medium with 7.5% DMSO; * — difference is significant compared to the corresponding control group without Trolox addition, $p < 0.05$; M ± SE

of survival rate as compared to 2.5% DMSO (up to 62.6% in Trolox-containing samples). Significant differences were recorded already at an antioxidant concentration of 20 μM in the sample. The maximum increase in survival rate (by 22%) was in the samples with 50—70 μM Trolox. In those cryopreserved with 7.5% DMSO, the maximum cell survival (up to 74%) was observed at Trolox concentrations of 50—200 μM.

Thus, the results obtained demonstrate the survival rate of cord blood HPCs to slightly exceed that of cord blood NCs, especially at optimal concentrations of cryoprotectant and antioxidant. This fact testifies to their higher resistance to cryogenic effects.

The analysis of the viability of cord blood NCs, including HPCs, using flow cytometry revealed no significant changes among all the studied experimental groups (Table 2). This parameter was about 75 and 65% for HPCs and NCs, respectively. Since these results do not provide a complete picture of the number of viable cells remaining in the samples after cryopreservation and subsequent *in vitro* transfusion simulation, and do not take into account the cell survival, the yield of viable $CD34^+$ $7AAD^-$- and $CD45^+7AAD^-$-cells was determined. This approach enables considering both the relative cell viability (described above) and absolute cell count in the sample, which can be used to determine effective cryoprotective solutions capable of stabilizing cells and improving their resistance to cryopreservation factors.

The findings showed that the yield of viable $CD45^+$-cells after cryopreservation in solutions with 2.5 and 5% DMSO and Trolox of different concentrations, as well as *in vitro* transfusion simulation, only tended to increase as compared to the control. It should be noted that cryopreservation in solutions with 5% DMSO provided a significantly higher (on average by 35%) yield of viable cells compared to 2.5% DMSO (Fig. 4). The analysis of the yield of viable cells in the samples with 7.5% DMSO and Trolox demonstrated the introduction of antioxidant at a concentration of 5—30 μM to improve this parameter, but the data did not differ significantly from the control. At the same time, concentrations of Trolox ranging from 50—200 μM provided a significant increase in the yield of viable cells by 23% compared to the control (Fig. 4).

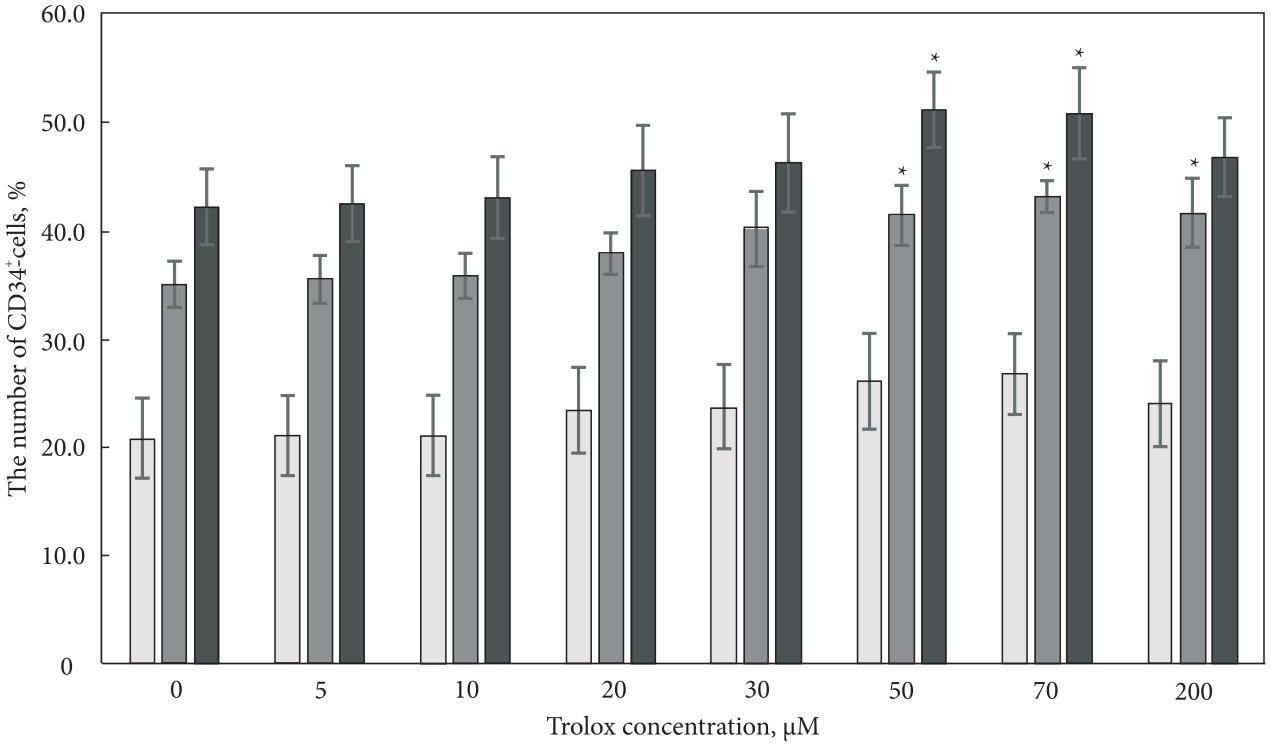

Fig. 5. Yield of viable CD34$^+$-cells after cryopreservation in solutions with DMSO and Trolox of different concentrations and *in vitro* transfusion simulation compared to their initial level before freezing: ▫ — cryoprotective medium with 2.5% DMSO; ▪ — cryoprotective medium with 5% DMSO; ■ — cryoprotective medium with 7.5% DMSO; * — difference is significant compared to the corresponding control group without Trolox addition, $p<0.05$; M ± SE

Pronounced changes in the yield of viable cells were observed in HPC analysis. While the introduction of Trolox at the tested concentrations into the samples with 2.5% DMSO caused no significant changes, the yield of viable HPCs in the samples cryopreserved with 5% DMSO was significantly higher compared to the control in the samples with 50—200 μM Trolox (Fig. 5).

A significant effect of Trolox supplement was also observed in the samples containing 7.5% DMSO and 50—70 μM antioxidant. In these samples, the yield of viable cells was 20% higher *vs.* the control, where no antioxidant was added. It is also worth noting that the yield of viable HPCs in the samples containing 7.5% DMSO was the highest and amounted to 55% in Trolox-containing media (50—70 μM). These findings suggest that the yield of viable HPCs after cryopreservation consistently exceeds the yield of CD45$^+$-cells, especially at optimal concentrations of cryoprotectant and antioxidant. This also indicates their higher cryoresistance, likely related to greater tolerance to osmotic changes and oxidative stress under freeze-thawing conditions.

Thus, the study confirmed the importance of a combined approach to cryopreservation of cord blood cells, using both the antioxidant Trolox and DMSO. This approach significantly improves the post-thaw survival and viability of cells, mitigates the negative effects of oxidative stress and opens up new prospects for enhancing the efficacy of cell therapy and regenerative medicine. The results obtained can be the basis for further optimization of long-term cell storage techniques for successful application in clinical practice.

CONCLUSIONS

Incubation of frozen-thawed cord blood NCs for one hour under physiological conditions *in vitro* reduces the survival and viability of NCs, including HPCs, compared to the data obtained immediately after thawing, that may be due to the destruction of cells with high ROS content.

It was found that cryopreservation of NCs using 7.5% DMSO and a water-soluble analogue of vitamin E: Trolox at a concentration of 50—70 μM reduced the number of cells with high ROS content and enabled the preservation of 13—25% more viable NCs, including HPCs, after 1-hour incubation under physiological conditions *in vitro* compared to the control samples, where no antioxidant was added.

These findings can be used to improve the current approaches for calculating the effective dose of cord blood cell preparations by estimating the cell loss rate when administered to the recipient's body.

REFERENCES

1. Allan DS. Using umbilical cord blood for regenerative therapy: proof or promise? Stem Cells. 2020; 38(5): 590-5.
2. Babijchuk LO, Gryschenko VI. Gurina TM, et al. inventors; Institute for Problems of Cryobiology and Cryomedicine, assignee. [The method of cryopreservation of cord blood nucleated cells, including hematopoietic stem cells]. Patent of Ukraine 92227, 2010 October 11. Ukrainian.
3. Baust JM, Vogel MJ, Buskirk RV, et al. A molecular basis of cryopreservation failure and its modulation to improve cell survival. Cell Transplant. 2001; 10: 561—71.
4. Berglund S, Magalhaes I, Gaballa A, et al. Advances in umbilical cord blood cell therapy: the present and the future. Expert Opin Biol Ther. 2017; 17(6): 691—9.
5. Davis JM, editor. Basic cell culture. A practical approach. Oxford: Oxford University Press; 2002. 382 p.
6. Distelmaier F, Valsecchi F, Forkink M. Trolox-sensitive reactive oxygen species regulate mitochondrial morphology, oxidative phosphorylation. Antioxid Redox Sign. 2012; 17: 1652—69.
7. Fry LJ, Querol S, Gomez SG, et al. Assessing the toxic effects of DMSO on cord blood to determine exposure time limits and the optimum concentration for cryopreservation. Vox Sang. 2015; 109(2): 181—90.
8. Giordano ME, Caricato R, Lionetto MG. Concentration dependence of the antioxidant and prooxidant activity of trolox in HeLa cells: involvement in the induction of apoptotic volume decrease. Antioxidants (Basel). 2020; 9: 1058—69.
9. Henson PM, Bratton DL, Fadok VA. The phosphatidylserine receptor: a crucial molecular switch? Nat Rev Mol Cell Biol. 2001; 2(8): 627—33.
10. 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.
11. Kumar A, Prasad JK, Srivastava N, Ghosh SK. Strategies to minimize various stress-related freeze-thaw damages during conventional cryopreservation of mammalian spermatozoa. Biopreserv Biobank. 2019; 17(6): 603—12.
12. Mancías-Guerra C, Sánchez-García S, Carreño-Salcedo S, et al. Dimethyl sulfoxide toxicity in umbilical cord blood transplantation in patients less than 4.5 kilos of weigh. Hematol Transfus Cell Ther. 2023; 45(1): 106—9.
13. Murugesan M, Nair CK, Nayanar SK, Pentapati KC. Flow cytometric enumeration of $CD34^+$ hematopoietic stem cells: A comparison between single- versus dual-platform methodology using the ISHAGE protocol. Asian J Transfus Sci. 2019; 13(1): 43—6.
14. Nekoonam S, Nashtaei MS, Naji M, et al. Effect of Trolox on sperm quality in normozospermia and oligozospermia during cryopreservation. Cryobiology. 2016; 72(2): 106—11.
15. Pushkar MS, Belous AM. [Introduction to cryobiology]. Kyiv: Naukova dumka; 1975. 343 p. Russian
16. Pushkar MS, Kapreliants OS, Pankov YeYa. [Cell ultrastructure at low temperatures.] Kyiv: Naukova dumka; 1978. 140 p. Russian
17. Rhee SG, Chang TS, Jeong W, Kang D. Methods for detection and measurement of hydrogen peroxide inside and outside of cells. Mol Cells. 2010; 29(6): 539—49.
18. Scott KL, Lecal J, Acker JP. Biopreservation of red blood cells: past, present and future. Transfus Med. Rev. 2005; 19(2): 127—42.
19. Sun JM, Kurtzberg J. Cord blood for brain injury. Cytotherapy. 2015; 17(6): 775—85.
20. Varo-Ghiuru F, Miclea I, Hettig A, et al. Lutein, Trolox, ascorbic acid and combination of trolox with ascorbic acid can improve boar semen quality during cryopreservation. Cryo Letters. 2015; 36(1): 1—7.
21. Zubov PM, Zubova OL, Babijchuk LO. Trolox antioxidant as a factor in stabilization of human cord blood nucleated cells during cryopreservation. Probl Cryobiol Cryomed. 2023; 33(2): 122—32.

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АНТИОКСИДАНТНИЙ ЗАХИСТ ПРИ КРІОКОНСЕРВУВАННІ:
РОЛЬ ТРОЛОКСУ В ЗБЕРЕЖЕННІ ЯДРОВМІСНИХ КЛІТИН КОРДОВОЇ КРОВІ ЛЮДИНИ

У роботі представлено експериментальні дані з визначення кількісних та якісних характеристик ядровмісних клітин (ЯВК), у тому числі гемопоетичних прогеніторних клітин (ГПК), кордової крові (КК) людини після

кріоконсервування в розчинах із різною концентрацією ДМСО та антиоксиданта тролоксу і моделювання трансфузії *in vitro*. Результати дослідження показали, що інкубація деконсервованих ЯВК КК впродовж години в фізіологічних умовах *in vitro* викликала зниження показників їх збереженості та життєздатності порівняно з даними, отриманими одразу після розморожування. Ці зміни, ймовірно, пов'язані з руйнуванням частини клітин, які містили високий вміст активних форм кисню (АФК), через механічні та метаболічні порушення, викликані факторами заморожування-відігрівання. Додавання на етапі еквілібрації в кріозахисне ДМСО-вмісне середовище антиоксиданта тролоксу в концентраціях 50 або 70 мкМ сприяє зменшенню кількості клітин із надлишковим вмістом АФК та підвищенню показників їх збереженості та життєздатності як після розморожування суспензії клітин, так і після перенесення до фізіологічних умов *in vitro*. Отримані результати з визначення відстроченої виживаності клітин вказують на ефективність застосування антиоксиданту тролоксу та перспективність розробки тролокс-вмісних кріопротекторних сумішей для заморожування ядровмісних, у тому числі ГПК, клітин КК людини та можуть стати передумовою для вдосконалення чинних та розробки но-вих підходів їх кріоконсервування та довгострокового зберігання в умовах низькотемпературного банка.

***Ключові слова*:** кріоконсервування, диметилсульфоксид, кордова кров, ядровмісні клітини, антиоксидант тролокс, моделювання трансфузії *in vitro*.