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COMPARATIVE ANALYSIS OF LIPID PEROXIDATION ACTIVITY IN SUSPENSIONS OF CRYOPRESERVED CORD BLOOD NUCLEAR CELLS UNDER EXPOSURE TO ANTIOXIDANTS - MEMBRANOPROTECTORS WITH DIFFERENT ACTION MECHANISMS

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ABSTRACT

An in-depth study of the oxidative homeostasis state into cell suspensions that contain hematopoietic stem cells is one of the key points for understanding ways to improve technologies for long-term storage of this material. Compounds with antioxidant action are considered promising additional cryoprotectants. Intensification of lipid peroxidation processes is one of the main factors causing disturbances in the barrier properties of cell membranes. Comparative analysis of changes in lipid peroxidation parameters during the cryopreservation-deconservation cycle showed that antioxidants-membrane protectors with different mechanisms of action (B-complex vitamins; α-lipoic acid, thiazotic acid morpholinium salt, 2-ethyl-6-methyl-3-hydroxy pyridine succinate) have similar features of a positive effect on the oxidative status of umbilical cord blood nuclear cell suspensions during cryopreservation. However, 2-ethyl-6-methyl-3-hydroxy pyridine succinate has a statistically significant advantage over α-lipoic acid and thiazotic acid morpholinium salt in terms of the conjugate formation dynamics during phospholipid peroxidation, which can be associated with its direct antioxidant effect.

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Introduction. In the last 25 years, human umbilical cord blood (UCB) has become widespread as a source of hematopoietic stem cells [1]. The provision of clinical needs in this material is carried out mainly through public low-temperature banks, where the UCB is stored in a cryopreserved state at the liquid nitrogen temperature [2].

It has been repeatedly shown that the formation of reactive oxygen species is one of the additional factors of cell damage during low-temperature storage [3]. In particular, it is believed that oxidative stress triggers mitochondrial or internal apoptotic mechanisms of cell death [4]. There are intensive biomolecule cell damages, uncontrolled release of calcium ions into the cytoplasm, disruption of enzyme systems, difficulties in conducting signals, and so on. Further accumulation of degradation products leads to an increase in membrane pathochemical and pathophysiological processes [5, 6]. The intensification of the processes of free radical oxidation leads to the enhanced formation of highly toxic substances, in particular, lipid peroxidation (LPO) products [7]. Simultaneous determination of intermediate and final molecular LPO product levels provides information about both the depth of violations and possible ways of their correction.

Aim. Carry out a comparative analysis of the safety of UCB nuclear cells (NCs) on the indicators of peroxidation of neutral lipids and phospholipids when using antioxidant-membraneprotective compounds (AOM) for cryopreservation.

Materials and methods. Human umbilical cord blood units were seized after obtaining prior informed consent and during physiological delivery. The method of collection in a "closed" system using a double plastic blood bags (250/100 ml (Baxter)) with a stabilizing solution CPDA-1 in a volume of 22 ml was used to obtain UCB units. Containers were stored in controlled temperature conditions ((21.5±3.5) °C) for no more than 24 hours. The fraction of UCB NC before and after its freezing (n = 44) was the object of research. Dimethyl sulfoxide (DMSO, Sigma, USA) was used as the main cryoprotective agent in a final concentration of 5%. A number of AOM in pharmacological form for intravenous use (B-complex vitamins (B1, B6 and B12) (Janssen Pharmaceutica, Belgium); α-lipoic acid (JSC "Farmak", Ukraine); thiazotic acid morpholinium salt (Halychpharm, Ukraine); 2-ethyl-6-methyl-3-hydroxypyridine succinate (JSC Lekhim, Ukraine)) was used for comparative studies on methods of UCB NCs preparation for freezing. UCB samples were divided into groups (I, II, III, IV) according to the used antioxidant compound. In all cases, the following treatment regimens were used: 8 μl of AOM per 10 ml of suspension was added 10 min before cryoprotectant (DMSO). The material was frozen in cryotubes with a volume of 4.5 ml at a rate of 1.0 ± 0.5 °C/min from a temperature of 10 °C to -156 °C (according to the technology of the State Institution "Institute of Hematology and Transfusiology of the National Academy of Medical Sciences of Ukraine" [8]). Defrosting was carried out in a water bath at a temperature of 38.0 ± 0.5 °C.

The activity of lipid peroxidation (LPO) processes was investigated using the method of I.A. Volchegorsky et al. [9] in our modification [10]. The optical density of the lipid extract was measured on a spectrophotometer Helios α (England). The method is characterized by differentiated determination of acyl peroxidation in the structure of the phospholipids (extracted to the isopropanol phase) and the unesterified intermediates of the neutralized lipids under fatty acid peroxidation (extracted to the heptane phase) according to the concentration of dienic conjugates (DC), trienic conjugates (TC), oxodienic conjugates (ODC) and final products by type Schiff basics (ShB) and substrates of the lipid peroxidation (the content of isolated double bonds (IDB). The LPO indicators are calculated in re-count of the NC content in 1 ml of suspension (accordingly, units on $1 \cdot 10^6$ NCs).

Statistical processing and data analysis were performed using STATISTICA 10 (StatSoft, USA). Since the distribution of the trait was not normal, a one-way nonparametric analysis of variance (ANOVA) was applied for independent groups - the Kruskal-Wallis method (Kruskal-Wallis ANOVA). If the null hypothesis of the absence of differences was rejected, a pairwise comparison of groups was performed using the nonparametric Mann-Whitney test. The Bonferroni correction (p = 0.00833) was used to overcome the so-called multiple comparison problem [11].

Results and its discussion. Four groups of UCB NCs suspensions were compared in terms of the AOM efficiency. The drugs were used in one concentration, which was determined as the best in previous studies on cryopreservation [12]. Analysis of the obtained data using the Kruskal-Wallis ANOVA showed that the studied groups of cell suspensions, which were under the influence of four different AOMs, did not have a statistically significant difference for most LPO indicators in the ratios before (a) and after (b) thawing (a / b) (Table 1, Table 2).

Table 1. The ratio of neutral lipids peroxidation before (a) and after (b) freezing with the addition of the studied antioxidants

Groups		Indicators				
		a/b IDB	a/b DC	a/b TC*	a/b ODC	a/b ShB
I	Me	1,070	1,128	1,063	1,040	1,017
	25%	1,047	0,883	0,796	0,791	0,810
	75%	1,318	1,506	2,833	2,722	2,967
	95% CI_{LB}	1,032	0,876	0,769	0,759	0,722
	95% CI_{UB}	1,377	1,506	2,833	2,722	3,810
II	Me	0,872	0,730	0,444	0,454	0,834
	25%	0,723	0,5664	0,319	0,314	0,741
	75%	0,997	0,953	0,971	0,947	1,199
	95% CI_{LB}	0,662	0,533	0,301	0,302	0,710
	95% CI_{UB}	1,055	0,998	1,081	1,019	0,818
III	Me	0,806	0,378	0,317	0,232	0,670
	25%	0,679	0,248	0,225	0,182	0,579
	75%	1,431	1,682	1,717	1,630	1,080
	95% CI_{LB}	0,461	0,236	0,176	0,150	0,383
	95% CI_{UB}	1,431	1,682	1,769	1,630	1,080
IV	Me	0,951	0,952	1,000	0,929	0,839
	25%	0,743	0,669	0,602	0,603	0,616
	75%	1,402	1,669	1,852	1,671	1,051
	95% CI_{LB}	0,799	0,756	0,689	0,732	0,696
	95% CI_{UB}	1,251	1,195	1,294	1,037	1,000

Me – Median; CI – confidence interval ($_{LB}$ - lower bound; $_{UB}$ - upper bound; (a/b) - ratio of indicators before (a) and after (b) freezing; *Kruskal-Wallis test – KW-H(3;56) = 8,57976084; p = 0,0354.

Table 2. The ratio of phospholipids peroxidation before (a) and after (b) freezing with the addition of the studied antioxidants

Groups		Indicators				
		a/b IDB	a/b DC	a/b TC*	a/b ODC	a/b ShB
I	Me	0,999	0,987	0,993	0,994	0,875
	25%	0,944	0,968	0,850	0,866	0,636
	75%	1,033	1,122	1,030	1,061	1,100
	95% CI_{LB}	0,908	0,968	0,824	0,832	0,635
	95% CI_{UB}	1,043	1,220	1,030	1,061	1,264
II	Me	0,964	0,978	0,897[1]	0,898	0,771
	25%	0,948	0,935	0,853	0,770	0,576
	75%	0,977	1,111	0,964	0,949	0,962
	95% CI_{LB}	0,947	0,935	0,841	0,740	0,505
	95% CI_{UB}	0,981	1,130	0,984	0,950	1,015
III	Me	0,9856	1,021	0,915[2]	0,880	0,733
	25%	0,643	0,928	0,792	0,700	0,623
	75%	0,990	1,096	1,055	1,093	0,870
	95% CI_{LB}	0,936	0,853	0,674	0,627	0,421
	95% CI_{UB}	1,007	1,095	1,079	1,093	0,457
IV	Me	0,956	1,043	1,097	0,900	0,716
	25%	0,877	0,992	1,050	0,750	0,470
	75%	1,290	1,242	1,334	1,058	1,110
	95% CI_{LB}	0,888	1,001	1,052	0,828	0,515
	95% CI_{UB}	1,195	1,199	1,225	1,039	1,027

Me – Median; CI – confidence interval ($_{LB}$ - lower bound; $_{UB}$ - upper bound; (a/b) - ratio of indicators before (a) and after (b) freezing; *Kruskal-Wallis test – KW-H(3;56) = 15.8305697; p = 0.0012; [1,2] - Mann-Whitney test: the difference between IV and II (p = 0.00075) IV and III (p = 0.00605) groups.

Rank analysis of variance revealed a statistically significant ($p < 0.05$) intergroup difference only for TC indices, and in both studied fractions (neutral lipids (TCn, Table 1) and phospholipids (TCph, Table 2, Fig.). In contrast to the other three AOMs, the addition of 2-ethyl-6-methyl-3-hydroxypyridine succinate (group IV) promoted a decrease in the level of DC and TC after thawing of cell suspensions. At the same time, after refinement by the Mann-Whitney test with Bonferroni correction (with a significance level of $p < 0.00833$), the intergroup difference in the ratio (a/b) was statistically confirmed only in the case of phospholipid peroxidation.

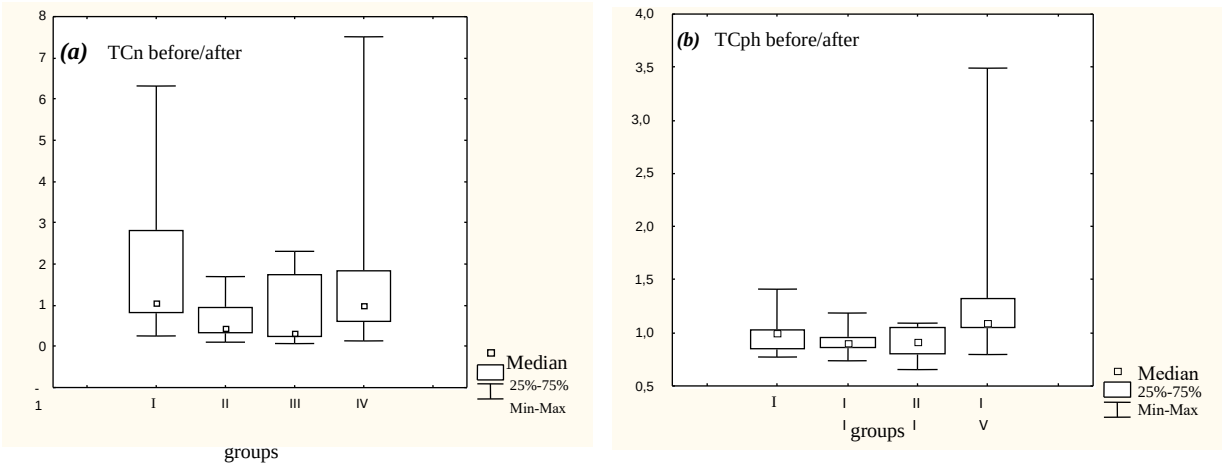

Fig. 1. Range charts by comparison group. The ratio of the content of triene conjugates (TC) before and after freezing depending on the antioxidant: (a) - TCn (with neutral lipids peroxidation), Kruskal-Wallis test: KW-H (3; 56) = 8.57976084; p = 0.0354; (b) – TCph (with phospholipids peroxidation, KW-H (3; 56) = 15.8305697; p = 0.0012.

An increase in the loss of hematopoietic cells due to the accumulation of free radicals during cryopreservation has been previously reported. The intensification of free radical oxidation reactions leads to the initiation of apoptosis with the onset of structural and functional changes in cell membranes up to cell death [13, 14]. Significant activation of lipid peroxidation occurs during freezing-thawing. When the cryoprotectant DMSO is used, LPO initiation is associated with the activity of secondary radicals of this substance [15]. The greatest changes were recorded at the stage of introducing the cryoprotectant DMSO into the NCs suspension, which upsets the balance of pro- and antioxidants [16]. Therefore, additional protection is the treatment of cells at the stage of preparation for cryopreservation with compounds that have antioxidant and membraneprotective effects.

An in-depth comparative analysis of the use effectiveness of antioxidants-membraneprotectors with different action mechanisms (including B-complex vitamins, α-lipoic acid, thiazotic acid morpholinium salt, and 2-ethyl-6-methyl-3-hydroxy pyridine succinate) in the same concentrations at the preparatory stage for cryopreservation showed that these substances were similar in their influencing on the oxidative status of UCB NCs suspensions. At the same time, the dynamics of the conjugates formation demonstrated the relative advantage of one among the used compounds, namely 2-ethyl-6-methyl-3-hydroxy pyridine succinate, as such, which had a direct mechanism of antioxidant action in combination with the activation of key enzyme antioxidant protection (glutathione reductase and glutathione peroxidase) [17, 18].

Conclusions. A number of membrane-protective antioxidants (B vitamins complex; α-lipoic acid, thiazotic acid morpholinium salt, 2-ethyl-6-methyl-3-hydroxy pyridine succinate) have a similar positive effect on lipid peroxidation processes. However, based on the dynamics of changes in the concentrations of diene and triene conjugates in the phospholipid fraction during cryopreservation, certain advantages of the direct-acting antioxidant 2-ethyl-6-methyl-3-hydroxy pyridine succinate have been established.

Declaration of interest statement. No conflict of interest exists.

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