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ENERGY METABOLISM OF PACKED WHITE CELLS AFTER CRYOPRESERVATION AND REHABILITATION IN A MEDIUM CONTAINING A CORD BLOOD LOW-MOLECULAR FRACTION

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To study the bioenergy indicators of leukoconcentrate cells after cryopreservation and possibility of their recovery after incubation in a medium with a low-molecular fraction from cow cord blood were the aim of work. Leukoconcentrate was obtained from donor blood by sedimentation; cryopreservation was carried out in the slow freezing regimen with 5% dimethylacetamide; amount of ATP, ADP and AMP was determined by chemiluminescence; glycogen — by cytochemical method. Evidence of energy disbalance of leukoconcentrate cells after cryopreservation was obtained. It was shown that the cord blood low-molecular fraction (0.15 mg/ml) activated glycogenolysis and increased contents of ATP, ADP and AMP in leukoconcentrate cells after cryopreservation. It was also shown that the cord blood low-molecular fraction contained energy substrates and metabolites, hormones and macro- and micronutrients. Use of the low-molecular fraction (below 5 kDa) from cord blood as a component of rehabilitating medium for frozen-thawed leukoconcentrate cells contributed to improvement of their energy status, which was manifested as augmentation in the total adenylate pool and glycogenolysis activation.

Key words: leukoconcentrat, cryopreservation, energy metabolism, cord blood.

Current methods of low-temperature storage of packed white cells cannot guarantee their high safety, which is due to heterogeneity of cell population in terms of composition and functional characteristics and, therefore, difficulty of unification of optimal cryopreservation conditions. The experimental research conducted to date both in Ukraine and abroad aimed at finding of better methods of leukocytes cryopreservation [1–5], which requires separation of packed white cells into individual populations and are not always clinically justified. Despite some progress, frozen-thawed leukocytes need to recovery of their functional status before clinical application. It is known that in leukocytes intensity bioenergetic processes changes after cryopreservation, it leads to abnormalities in energy-dependent reactions [6, 7] and hence to impairment of their functional activity.

Recovery from cryoinjuries, first of all, requires energy costs, which can be replenished via glycolysis and oxidative phosphorylation.

To solve this problem, use of special rehabilitating media capable of stimulating energy generation processes is promising.

We present the results of studying energy metabolism of donor blood packed white cells cryopreserved with dimethylacetamide. Evidence of a stimulating effect of a low-molecular fraction from cow cord blood (CBF) on ATP, ADP and AMP contents and glycolysis in the test cells is adduced.

Materials and Methods

Obtaining leukocyte mass

Leukoconcentrate was obtained from human donor blood by sedimentation of erythrocytes in 6% dextran solution (m.m. 50,000–70,000, “Biokhim”, Russia) [8].

Cryopreservation of leukocyte mass

Cryopreservation of cell suspension samples (0.5 mL, 2×10^7 cells/ml) was carried out according to the following regimen of slow freezing with 5% dimethylacetamide (DMAc):

at the rate of 3 °C/min from 22 °C to the crystallization point (−16 °C) with a shutdown of process for 6.5 min; then at the rate of 5 °C/min to −100 °C followed by immersion of samples in liquid nitrogen (−196 °C) [9]. Suspension was thawed in a water bath at 38 °C with intensive shaking containers [10]. After thawing the cryoprotectant was removed by slow dilution of cell suspension samples with autoplasm containing 2% dextran (1:2) followed by centrifugation (7 min at 200 g) [11].

Integrity of Leukocytes

The leukocyte integrity was assessed by trypan blue exclusion test [12].

Determination of ATP, ADP and AMP Contents

Cell suspension samples (0.5 ml) were incubated at 37 °C for 20 min with or without CBF (0.15 mg/ml). The incubation time was based on the experimental studies of granulocyte energy metabolism, viz., ATP and lactate production rates [13, 14]. After incubation adenylates were extracted.

Adenylates were extracted from cells by boiling leukocyte suspension samples (10⁷ cells/ml) in Tris-buffer. 3 ml of boiling 0.1 M Tris-0.01M EDTA-acetate buffer (pH = 7.75) were added to an aliquot of cell suspension (0.5 ml); the aliquot was exposed in a boiling water bath for 5 min. Extracts were cooled down to the ambient temperature and stored at −18 °C for further analytic procedures.

ATP content in the resulting extract was determined by a chemiluminescence method [14] using a firefly enzyme, luciferase, and luciferin (Sigma, USA) (ATP Luminometer — 1250, LKB, Sweden). The maximum values of light reaction were registered.

To calibrate dependence of light reaction on ATP concentration, values of light emission of standard ATP solutions obtained at the beginning and end of an experimental series were used. To match light reaction values with ATP concentrations readings of light emission of ATP standard solutions were obtained at the beginning and end of each series of runs. ATP concentrations were calculated on the basis of relations of light impulse values to ATP standard solution concentrations adjusted for dilution, extract volumes and sample weights equivalent to protein. Background adenylate content in cell-free medium was also accounted in the calculations.

ADP and AMP contents were determined via preliminary restitution of these nucleotides to ATP using phosphoenolpyruvate (Sigma, USA), pyruvate kinase (Sigma, USA), and myokinase (Sigma, USA). The amount of synthesized ATP was again estimated by chemiluminescence assay. Adenylic nucleotide concentrations were converted to molar unit.

Calculation of Adenylic Pool: adenylic pool is the sum of ATP, ADP and AMP concentrations.

Calculation of Adenylic Energy Charge: adenylic energy charge (AEC) was calculated by the following formula [15]:

$$AEC = \frac{C_{ATP} + \frac{1}{2} C_{ADP}}{C_{ATP} + C_{ADP} + C_{AMP}}.$$

AEC characterizes the state of adenylic system charge; for example, if the whole pool of adenylic nucleotides is purely represented by ATP (maximum of high-energy bonds) AEC equals one; if there is only AMP in the cell (no high-energy bonds) AEC equals zero [15].

Determination of Glycogen Content

Glycogen in leukoconcentrate neutrophil cytoplasm was analyzed by cytochemically method [16] using Schiff reagent (PAS reaction) (Merck, Germany). Before the test leukoconcentrated was washed out by centrifuging (7 min at 200 g) in glucose-free medium of the following composition: 5% bovine serum albumin (Serva, Germany) in physiologic saline, 10% dextran in physiologic saline (“Reopoliglyukin-Novofarm”®, Novofarm-Biosynthesis, Ukraine). The ration leukocyte suspension/dextran/albumin was 1:1:1. Supernatant was removed; precipitate was resuspended in fresh solution. Glycogen content was semiquantitatively estimated with calculation of the average cytochemical coefficient (ACC) according to Kaplow [16]: $ACC = (3A + 2B + C) / 100$, where A — number of cells with strong positive staining, B — number of cells with staining of medium intensity, C — number of cells with weak positive staining and/or negative staining. To inhibit glycolysis, sodium iodoacetate was used at the final concentration of 1 mM [17]. To activate cells, packed white cells were incubated for 1 hour at 37 °C after inoculation of one-day culture of *Staphylococcus aureus* strain 209 in physiologic saline (inactivated by 1-hour exposure in a water bath at 80 °C) at the concentration of ~2 bln cells/ml by the turbidity standard.

CBF (below 5 kDa) Extracting

The extraction of the fraction containing components with molecular weights below 5 kDa from the cryodestructed whole cattle blood was performed by ultrafiltration [18] using a membrane module Sartorius (Germany). Ultrafiltrate was lyophilized in a freeze-drying chamber under the pressure of 5×10^{−2} mmHg; the total duration of drying was 28–30 h. Lyophilized samples

were stored at -80°C . CBF was added to the incubation medium at the final concentration of 0.15 mg/ml .

Determination of CBF Composition

CBF was assessed for estradiol, free triiodothyronine, cortisol, and progesterone contents using a closed test system for quantitative analysis of hormones *in vitro* by electrochemiluminescent immunoassay (ECLIA) on an automatic Elecsys 1070/2010 analyzer and a Modular Analytics E170 analyzer (Hoffmann-La Roche, Switzerland). To estimate micro/macroelements and metabolite concentrations (calcium, magnesium, phosphorus, zinc, glucose, creatinine, etc.) a closed test system for quantitative analysis of substances in biological liquids by electrochemiluminescent assay and automatic Elecsys 1070/2010 and Cobas C311 chemistry analyzers were used (Hoffmann-La Roche, Switzerland).

Inhibitory Assay

To explain the mechanism of action of CBF on energy processes native leukoconcentrate suspension was pre-incubated with a glycolysis inhibitor, sodium iodoacetate (IA) (Serva, Germany), which blocks one of the key glycolytic enzymes — glyceraldehyde-3-phosphate dehydrogenase at 37°C for 20 min. IA was added to leukocyte suspension (10^7 cells/ml) before CBF at the final concentration of 1 mM [17].

The non-parametric Mann–Whitney test was used for statistical analysis.

Results and Discussion

The analysis of safety of native packed white cells obtained from human blood by sedimentation showed that suspension had $97.81\pm0.42\%$ of viable nucleated cells.

After cryopreservation with DMAc at the final concentration of 5% the number of provisionally viable cells in samples was $79.96\pm1.75\%$.

To understand changes occurring in the adenylic energy system after cryopreservation of cells, we assessed ATP, ADP and AMP contents, the total adenylic pool, adenylic energy charge and ratios between individual adenylic nucleotides.

The investigation of the baseline energy status of packed white cells revealed that they had the full set of adenylic nucleotides represented by ATP, ADP and AMP. AEC of freshly-isolated cells was 0.79 ± 0.003 rel. u., which suggests predominance of ATP in the total adenylic pool and, therefore, abundance of energy in the adenylic system of cells (Table 1). The data in Table 1 show that after cryopreservation balance of the cell energy system is impaired.

Thus, it was demonstrated that the total adenylic pool significantly reduced mainly due to the decrease in ATP content by 2.6 times (23.42 ± 2.9 vs. 8.9 ± 0.93 nmol/mg protein). It is noteworthy that the drastic drop in ATP content in frozen-thawed cells was not accompanied by expected rise in relative concentrations of ADP and AMP via their interconversion, that is why the ration ATP:ADP:AMP considerably shifted to di- and monophosphates. We assume that this can be attributed to loss of adenylic nucleotides through transmembrane pores that form during cryopreservation and capable for self-healing [19]. It is important to note that the selected method of cryopreservation, despite the distortion in ratios between individual adenylic nucleotides, provides preserving AEC on the level of 0.64 ± 0.070 rel. u.

Table 1. Characterization of General Status of the Adenylic System of Packed Human White Cells

Index	Before cryopreservation	After cryopreservation	After cryopreservation + CBF
ATP, nmol/mg of protein	23.42 ± 2.9	8.9 ± 0.93 *	11.9 ± 0.68 **
ADP, nmol/mg of protein	9.68 ± 1.21	8.37 ± 2.6	16.87 ± 4.4 #
AMP, nmol/mg of protein	3.02 ± 0.39	4.6 ± 1.7	9.56 ± 2 #
Adenylic Pool, nmol/mg of protein	37.49 ± 4.09	21.47 ± 5.6 *	37.9 ± 5.9 #
AEC, rel. u.	0.79 ± 0.003	0.64 ± 0.070	0.56 ± 0.080
Ratio ATP: ADP: AMP	7.75: 3.2: 1	1.2: 1.8: 1	1: 1.8: 1

Footnotes: * — significant difference in comparison with the before-cryopreservation control ($P < 0.05$); # — significant difference in comparison with the after-cryopreservation control ($P < 0.05$).

It is known that in energy metabolism in cells of leukocytic series glycogen metabolism plays a key role, being used as energy substrate by cells. In particular, this refers to phagocytic types of leukocytes, which function under acute hypoxia in tissues, at the same time keeping a high level of metabolism [20]. Need of leukocytes in large stock of glycogen is predominantly related to a drastic increase of energy expenditure by stimulated cells. Given the above, at the next stage of our experimental work we performed cytochemical analysis of glycogen content in neutrophils from packed white cells after cryopreservation with DMac.

The data showed that freshly-isolated cells contain large amounts of PAS-positive matter, glycogen, which uniformly or as conglomerates and granules fills the whole cytoplasm. ACC was 2.139 ± 0.01 rel. u., which corresponded to the normal range (Fig. 1).

Further incubation of cells in glucose-free medium for 1 hour did not lead to visible changes in glycogen content in cytoplasm, consequently, no ACC changes were recorded (1.98 ± 0.02 rel. u.). Activation of freshly-isolated cells by inoculating *Staphylococcus aureus* suspension under these conditions did not promote glycogenolysis ($ACC = 1.9 \pm 0.02$ rel. u.). Analyzing these results, we can conclude that native neutrophils do not need mobilization of cytoplasmic stock of glycogen under these conditions, at least within 1-hour incubation. DMac exerted no impact on this parameter.

At the same time a completely different pattern was observed for glycogen content in neutrophils from packed white cells cryopreserved with 5% DMac. The glycogen amount in neutrophils from frozen-thawed

packed white cells remained unchanged in comparison with that in freshly-isolated cells: ACC was 1.93 ± 0.018 and 2.139 ± 0.01 , respectively (Fig. 2). However, after 1-hour incubation of quiescent cells a statistically significant decline in glycogen content was observed ($ACC 1.73 \pm 0.01$ rel. u.), which was seen in stained slides as weakening of staining intensity in cytoplasm.

Unlike freshly-isolated cells, phagocytizing neutrophils from frozen-thawed leukoconcentrate actively used glycogen stock, which was seen as changes in intensity and nature of cytoplasmic staining and a significant reduction in ACC to 1.66 ± 0.007 rel. u. (Fig. 2). Microscopic analysis of slides demonstrated decreased intensity of cytoplasmic staining, absence of glycogen conglomerates, and in slides PAS-negative neutrophils regularly occurred.

Thus, DMac-containing cryoprotective solution provides preserving sufficiently large numbers of viable cells, nevertheless, taking into account the above-mentioned data, we can conclude that leukoconcentrate quality after cryopreservation still worsens, and there is an unmet need in recovery of functional status of cells before transfusion to guarantee the best therapeutic benefit.

Previously we demonstrated that application of rehabilitating medium containing a low-molecular fraction (below 5 kDa) from cord blood contributed to recovery of functional status of frozen-thawed leukocytes, namely augmentation in parameters of their phagocytic activity [9, 21, 22]. All phagocytic stages are known to depend largely on energy supply of cells via glycolysis and oxidative phosphorylation, and in phagocytizing cells the contribution of glycolysis

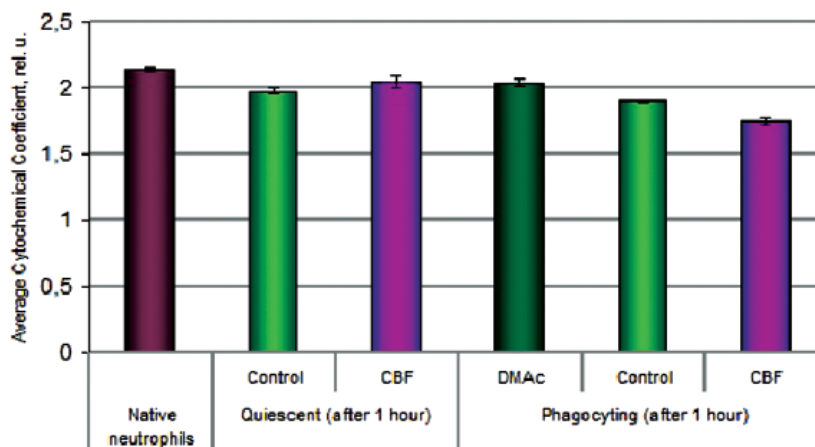


Fig. 1. Glycogen Content in Neutrophils from Packed Human White Cells prior to Cryopreservation Depending on Incubation Medium Components

in the total energy pool is significantly higher [13, 14]. Therefore, we assumed that mechanisms of the CBF stimulating effect were mediated through influence of its components on energy metabolism.

As one can see in Table 1, CBF contributed to increase in ATP, ADP and AMP concentrations in frozen-thawed cells by 1.34, 2 and 2 times, respectively, resulting in the gain in the total adenylic pool by 76.5%, which reached the level of freshly-isolated cells. However, CBF had no impact on AEC. It could be explained both by intensified ATP consumption due to activation of recovery processes aimed at elimination of injuries and by ADP and AMP *de novo* synthesis, since these both processes lead to a shift in ratios between individual adenylic nucleotides in favor of ADP and AMP.

To elucidate mechanisms of CBF action on the test parameters, we used glycolysis inhibitor, sodium iodoacetate, for freshly-isolated cells (Fig. 3). It was found that addition of sodium iodoacetate to incubation medium was associated with the decline in intracellular ATP by 1.7 times, however, as early as after 20-minute incubation of inhibitor-treated cells with CBF we noticed the

increase in this parameter by 1.4 times.

At the same time there was no return of ATP concentration to the level observed in CBF-treated cells without inhibitor: in Fig. 3 one can see that iodoacetate suppresses the stimulating effect of the fraction on ATP production by 1.9 times (35.42 ± 3.91 vs. 18.78 ± 2.05 nmol/mg protein). This indicates that in addition to glycolysis, other pathways of ATP synthesis can be influenced by low-molecular components of CBF.

The most conspicuous reduction in ACC was observed, when CBF was added to frozen-thawed quiescent (down to 1.61 ± 0.017 rel/ u.) and phagocytosing (down to 1.31 ± 0.005 rel. u.) cells (Fig. 2). This attests to the fact that CBF upon activation of phagocytes with cryopreservation-impaired metabolism assists mobilization of cytoplasmic glycolytic stock, thereby satisfying the need of cells in glucose, which is likely to be used for energy production in glycolytic reactions.

To confirm this assumption, we used inhibitor analysis with sodium iodoacetate again. Fig. 2 shows that treatment of cells with glycolysis inhibitor resulted in conservation of glycogen stock in cytoplasm — staining intensity was the same as in control, and ACC was 2.09 ± 0.02 rel. u. In CBF presence the blocking effect of inhibitor persisted;

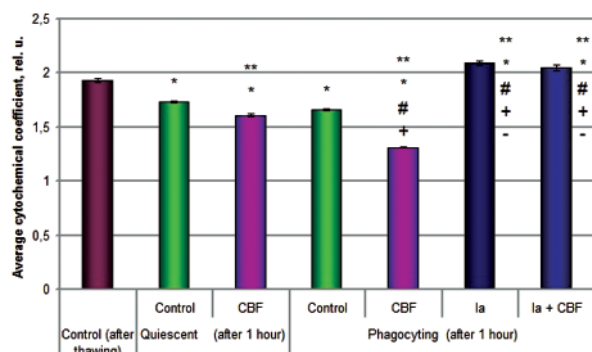


Fig. 2. Glycogen Content in Neutrophils from Packed Human White Cells after Cryopreservation Depending on Incubation Medium Components:

- * — significant difference in comparison with control after cryopreservation ($P < 0.05$);
- ** — significant difference in comparison with quiescent control ($P < 0.05$);
- # — significant difference in comparison with phagocytosing control ($P < 0.05$);
- + — significant difference in comparison with quiescent cells incubated with CBF ($P < 0.05$);
- — significant difference in comparison with phagocytosing cells incubated with CBF ($P < 0.05$).

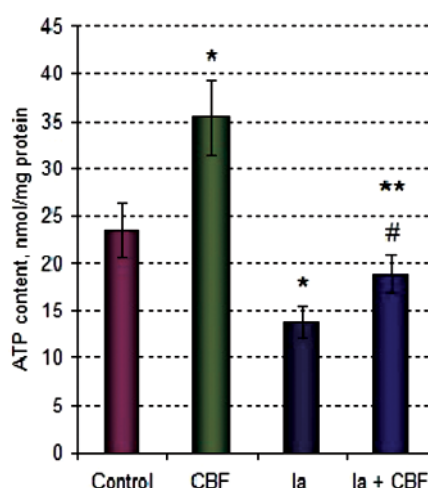


Fig. 3. Effect of CBF on ATP Content in Freshly-Isolated Packed White Cells Treated with Iodoacetate:

- * — significant difference in comparison with the before-cryopreservation control ($P < 0.05$);
- ** — significant difference in comparison with incubation of leukoconcentrate cells with Ia ($P < 0.05$);
- # — significant difference in comparison with incubation of leukoconcentrate cells with CBF ($P < 0.05$).

Table 2. Composition of the Cattle Cord Blood Low-Molecular (below 5 kDa) Fraction

Component	Content of CBF solution (1 mg/ml)
Estradiol	20.78 pg/ml
Free Triiodothyronine	0.76 pmol/L
Cortisol	0.415 nmol/L
Progesterone	0.03 ng/ml
Polypeptides	0.15 mg/ml
Calcium ionized	0.2 mmol/L
Magnesium	0.3 mmol/L
Phosphorus	0.195 mmol/L
Zinc	2.52 μ mol/L
Glucose	0.39 mmol/L
Lactate	0.15 mmol/L
Creatinine	154 μ mol/L
Chondroitin sulfates general	0.9 mg/L
Uronic acid	2.98 mg/L
Glycoproteins	25.3 mg/L
Total GAG	0.007 U
ATP	1,18 nmol/ml
ADP	0,818 nmol/ml
AMP	0,25 nmol/ml

staining intensity in cytoplasm and ACC (2.05 ± 0.029 rel. u.) did not change in comparison with the baseline values. These results confirm the fact that addition of CBF to incubation medium promotes glycogenolysis in frozen-thawed neutrophils, and glucose produced in this process is used in glycolytic reactions for further ATP synthesis.

Since CBF is isolated from cord blood rich in bioactive substances, one cannot exclude that its beneficial effect on cell energy status is attributed to energy substrates such as glucose, adenylic precursors or regulatory biomolecules contained in it. In view of this we analyzed CBF composition (Table 2).

The data summarized show that there are energy substances and metabolites represented by glucose, creatinine, lactate, ATP, ADP, and AMP as well as metabolism regulators represented by hormones, calcium ions and macro/microelements in the fraction. Currently, it turns to be impossible to ascribe the biological activity of CBF to a certain component; its mechanism of action is most likely to be due to a complex action of low-molecular substances.

Thus, the study results showed that DMAc as a component of protective solution for cryopreservation of packed white cells provides preserving sufficiently large numbers of viable cells. However, taking into consideration the data on impairment in bioenergy balance of cells, we can conclude that leukoconcentrate quality still worsens after cryopreservation.

Use of the low-molecular fraction (below 5 kDa) from cord blood as a component of rehabilitating medium for frozen-thawed leukoconcentrate cells contributed to improvement of their energy status, which was manifested as augmentation in the total adenylic pool and glycogenolysis activation.

Using glycolysis inhibition, we revealed that CBF effect on energy system of cells could be mediated at least by two mechanisms. First, it is possible that CBF low-molecular components are directly involved in glycolytic reactions, since glycolysis pathway of energy generation is the major one for cells of leukocytic series. Second, we cannot rule out CBF influence on other pathways of ATP synthesis, in particular on oxidative phosphorylation.

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**ЕНЕРГЕТИЧНИЙ ОБМІН
ДЕКОНСЕРВОВАНИХ КЛІТИН
ЛЕЙКОКОНЦЕНТРАТУ ПІСЛЯ
РЕАБІЛІТАЦІЇ В СЕРЕДОВИЩІ,
ЩО МІСТИТЬ НИЗЬКОМОЛЕКУЛЯРНУ
ФРАКЦІЮ КОРДОВОЇ КРОВІ**

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Метою роботи було визначити біоенергетичні показники клітин лейкоконцентрату після кріоконсервування та можливість їх відновлення після інкубації в середовищі з низькомолекулярною фракцією кордової крові. Лейкоконцентрат одержували з донорської крові методом седиментації; кріоконсервування проводили в режимі повільного заморожування під захистом 5% -го диметилацетаміду; кількість АТФ, АДФ і АМФ визначали хемолюмінесцентним, а глікоген — цитохімічним методами. Встановлено, що після кріоконсервування відбувається порушення енергетичного балансу клітин лейкоконцентрату. Показано, що низькомолекулярна фракція кордової крові (0,15 мг/мл) сприяє глікогенолізу і підвищенню вмісту АТФ, АДФ і АМФ в деконсервованих клітинах. До складу фракції кордової крові входять енергетичні субстрати й метаболіти, гормони, макро- та мікроелементи.

Використання фракції кордової крові у складі реабілітувального середовища для деконсервованих клітин лейкоконцентрату сприяє поліпшенню їхнього енергетичного статусу, що виявляється у збільшенні загального аденілатного пулу і активації процесів глікогенолізу.

Ключові слова: лейкоконцентрат, кріоконсервування, енергетичний обмін, кордова кров.

**ЭНЕРГЕТИЧЕСКИЙ ОБМЕН
ДЕКОНСЕРВИРОВАННЫХ КЛЕТОК
ЛЕЙКОКОНЦЕНТРАТА ПОСЛЕ
РЕАБИЛИТАЦИИ В СРЕДЕ,
СОДЕРЖАЩЕЙ НИЗЬКОМОЛЕКУЛЯРНУЮ
ФРАКЦИЮ КОРДОВОЙ КРОВИ**

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Целью работы было определение биоэнергетических показателей клеток лейкоконцентрата после кріоконсервирования и возможности их восстановления после инкубации в среде с низькомолекулярной фракцией кордовой крови. Лейкоконцентрат получали из донорской крови методом седиментации; кріоконсервирование проводили в режиме медленного замораживания под защитой 5% -го диметилацетамида; количество АТФ, АДФ и АМФ определяли хемолюминесцентным, а гликоген — цитохимическими методами. Установлено, что после кріоконсервирования происходит нарушение энергетического баланса клеток лейкоконцентрата. Показано, что низькомолекулярная фракция кордовой крови (0,15 мг/мл) способствует гликогенолизу и повышению содержания АТФ, АДФ и АМФ в деконсервированных клетках. В состав фракции кордовой крови входят энергетические субстраты и метаболиты, гормоны, макро- и микроэлементы.

Использование фракции кордовой крови в составе реабилитирующей среды для деконсервированных клеток лейкоконцентрата способствует улучшению их энергетического статуса, что выражается в увеличении общего аденилатного пула и активации процессов гликогенолиза.

Ключевые слова: лейкоконцентрат, кріоконсервирование, энергетический обмен, кордовая кровь.