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RESTORATION OF THE STRUCTURAL AND FUNCTIONAL STATE OF ERYTHROCYTES AFTER HYPOTHERMIC STORAGE USING HUMAN CORD BLOOD LOW-MOLECULAR FRACTION AND THE DRUG ACTOVEGIN

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One of the modern transfusiology tasks is to preserve the properties of erythrocytes after hypothermic storage. The transfusion medium quality and efficiency depend on their functional state. Plausible protective effects of a human cord blood low-molecular fraction (CBF) and the drug Actovegin were evaluated in the study in order to use them as components of recovery medium.

Aim. The purpose of this study was to investigate the CBF and the drug Actovegin effect on erythrocytes morphology, energy balance, and oxygen transport function of erythrocytes after their hypothermic storage.

Materials and methods. Erythrocyte mass was stored in hypothermia for 7–21 days. Every 7 days, the CBF or Actovegin (final concentration 0.6 mg/ml) were added to the samples and incubated for 1 h at 37 °C. CBF was obtained from human cord blood by ultrafiltration. The erythrocytes morphology was assessed using light microscopy. The content of ATP and 2.3-DPG was determined by photoelectrocalorimetric method. The saturation, O_2 and CO_2 tension were assessed using an analyzer of blood gases. The ratio of hemoglobin forms was studied by photometry.

Results. It has been shown that both CBF and the drug Actovegin helped to restore the morpho-functional characteristics of erythrocytes after 21 days of their storage at 2–4 °C. This was indicative as an increase in the normocytes number increase and restoration of O_2 tension, saturation, ATP and 2.3-DFG content, and normalization of the hemoglobin forms ratio. It is suggested that the mechanisms of the drags action was are associated with ATP synthesis activation and 2.3-DFG formation.

Conclusions. The use of media containing CBF or the drug Actovegin enabled to effectively restore the properties of erythrocytes disturbed after their prolonged storage.

Key words: cord blood, Actovegin, erythrocytes, hypothermia, recovery medium.

In practical transfusiology, preservation of donor blood erythrocytes under low temperatures and with special preservative solutions is widely used for their long-term storage. This method enables not only to provide strategic reserves of red blood cells, but also to accumulate several doses from one donor for transfusions under the scheme “one donor — one recipient” [1]. However, since the 70’s it is known that the violation of the main indicators of erythrocytes’oxygen transport function, such as saturation and ATP (adenosine triphosphate) and 2,3-DFG (2,3-diphosphoglycerate) content, occurs already on the third day of hypothermic storage (2–4 °C) [2]. Given this, the efforts of scientists and hematologists over the last decades were aimed at solving the problem of restoring the structural and functional integrity of erythrocytes after hypothermic storage, and much attention was paid to the methods for extending the storage period of thawed erythrocytes after cryopreservation [1, 3, 4]. To do this, numerous attempts

were made to develop hemoconservatives and rehabilitation media, which helped stabilize the membrane of deconserved erythrocytes due to non-penetrating components (sucrose, proteins, polymers) and increase their energy potential due to penetrating metabolites of carbon phosphate metabolism [5–9]. As a result of these studies, it was managed to extend the hypothermic storage period fordeconserved erythrocytes from 24 hours to 5–21 days, provided the use of the "closed systems" of preservation, hardware methods and additional solutions [1]. However, a common disadvantage of such additional solutions is that their components have usually a negative side effect on the recipient's body [6]. In addition, to obtain the desired effect, most of these media should be used in a ratio where the donor erythrocytes volume in the sample is less than the rehabilitation solutionvolume, which provoke additional stress in the recipient.

We have previously shown the possibility of using a low molecular weight fraction (up to 5 kDa) from cord blood or a similar pharmaceutical product Actovegin, which is known to be a low molecular weight blood fraction (up to 5 kDa) of dairy calves, to rehabilitate leukoconcentrate cells after cryopreservation. It has been proven that low molecular weight fraction (up to 5 kDa) from cord blood or the drug Actovegin, which is a deproteinized hemoderivative from dairy calves, can significantly increase the energy potential of cells, have a balanced composition of biomolecules and do not have toxic effects on the animals' body and different cell cultures [10–13]. Based on the above, the aim of this study was to investigate the restorative effect of low molecular weight fraction (up to 10 kDa) from human cord blood (CBF) and the drug Actovegin on the main indicators of erythrocytes'oxygen transport function after long-term hypothermic storage.

Materials and Methods

Ethical approval. Human cord and donor blood samples were collected and manipulated in accordance with the recommendations of the Helsinki Declaration of the World Medical Association for Biomedical Research on Humans (Helsinki, 1964, Somerset West, 1996), as well as the recommendations of the European Convention for the Vertebrates Protection used for research and other scientific purposes (Strasbourg, 1986).

The method of CBF obtaining. The low molecular weight fraction with molecular weight components up to 10 kDa was obtained from human cord blood after cryodestruction using the method of multistage ultrafiltration using equipment from Sartorius (Germany) [14]. Lyophilization of the ultrafiltrate was performed in a freeze-drier at a temperature of −40 °C for 18–20 hours under average pressure in a chamber of 102 Pa. Ready made CBF samples have the form of a hygroscopic substance of yellowish color. Before use, a drug portion was dissolved in sterile physiological solution (Lekhim-Kharkiv, Ukraine).

Scheme of experiment. Erythrocyte mass was obtained from the whole human donated blood preserved with the hemopreservative Glugicir or CPDA-1 at the Kharkiv Blood Transfusion Center by removing the leukothromocytelayer after centrifugation (3 000 g, 5 min). The obtained erythrocyte mass was stored under sterile conditions at a temperature of 2–4 °C. In control periods (1, 7, 14 and 21 days) aliquots of erythrocyte mass (950 µl) were taken and CBF (50 µl of solution to a final concentration in samples of 0.6 mg/ml) or the drug Actovegin for a comparative series of experiments (solution for injection, 40 mg/ml "Takeda" (Austria), the final concentration in the samples was 0.6 mg/ml) were added to them. 50 µl of sterile physiological solution (Lekhim-Kharkiv, Ukraine) was added to the control samples. Thus, 3 experimental groups were formed. The obtained samples were incubated for 1 hour at a temperature of 37 °C, after which further studies were performed.

S*tudy of erythrocyte morphology*. After incubation of erythrocyte mass samples with the studied preparations, clinical smears were prepared, which were fixed according to May-Grunwald and stained according to Romanovsky [15]. Light microscope (PZO-Warszava, Poland) under immersion (eyepiece 8, objective 100) was used for erythrocyte morphology analyze, which included counting cells in 10–12 fields of view (at least 1000 cells per smear) and determining the ratio of normocytes and echinocytes number.

ATP and 2,3-DFG content determining. The ATP and 2,3-DFG content was studied by non-enzymatic method evaluating inorganic phosphorus in erythrocyte hydrolysates. The amount of inorganic phosphorus was calculated by photoelectrocalorimetric method [16–18]. The amount of ATP and 2,3-DFG was monitored on the 7th, the 14th and the 21st

day of storage (2–4 °C). To do this, after each specified period of hypothermic storage, CBF or the drug Actovegin were added to the samples of erythrocyte mass at a final concentration of 0.6 mg/ml and incubated for 1 h at 37 °C. The concentration of ATP and 2,3-DFG was then measured. To determine the mechanism of the studied preparations action, before their addition the cells were additionally incubated with a glycolysis inhibitor sodium iodoacetate (Serva, Germany) for 5 min at 37 °C at a final concentration of 1 mM.

Parameters of erythrocytes oxygen transport function research. To assess the oxygen transport function of erythrocytes, the dynamics of changes in such indicators as saturation (percentage of oxygen in the blood) and oxygen and carbon dioxide tension in experimental samples of erythromass from human donor blood were analyzed using a cartridge analyzer IL GEM Premier — 3000.

Determination of hemoglobin forms ratio. To study the effect of CBF and the drug Actovegin on the hemoglobin forms ratio, erythromass from human donor blood was incubated with the studied drugs, as described above. After that, the samples were subjected to three-time washing with physiological solution at 3000 rpm for 15, 5 and 5 min, respectively. In the obtained erythrocyte mass, the ratio of hemoglobin forms was determined by the photometric method according to [19].

Statistical analysis of the obtained data was performed using the software package "StatGraphics Plus 2.1". Verification of the data distribution normality was performed according to the Shapiro-Wilk W-test. Student's t-test was used to compare two independent groups on one basis. The value of the significance level P was taken equal to 0.05, which meets the criteria adopted in biomedical studies. Data are presented as arithmetic mean ± standard deviation.

Results and Discussion

Study of changes in erythrocyte morphology. The effect of CBF and the drug Actovegin on the change in erythrocyte morphology after hypothermic storage was primarily studied. In control samples of donated blood, the number of normocytes decreased rapidly during the first 7 days of hypothermic storage from 80% to 21% (Fig. 1, *A*), while the number of echinocytes increased from 12% to 70% (Fig. 1, *B*). Incubation of cells in medium containing CBF or the comparison drug Actovegin for 1 h at 37 °C to the same extent and significantly contributed to the restoration of erythrocytes morphological parameters, most pronounced at the 21st day of hypothermic storage (Fig. 1).

Thus, the analysis of the obtained data showed that starting from the 7th day of hypothermic storage after incubation in a rehabilitation medium containing CBF (up

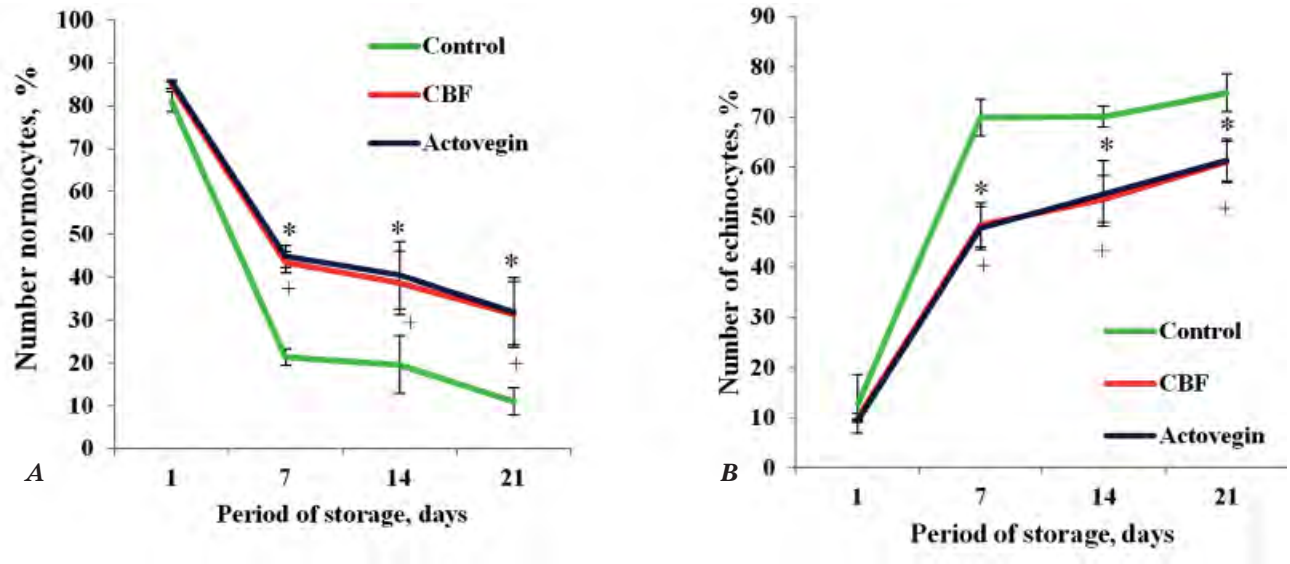

Fig. 1. **The effect of the human cord blood low-molecular fraction and the drug Actovegin on the number of normocytes (*A*) and echinocytes (*B*) in human donor blood depending on the duration of hypothermic storage**
*, + — differences are significant in comparison with control values in the corresponding day of hypothermic storage ($P \leq 0.05$); $n = 6$

to 10 kDa) or the drug Actovegin, there was a significant improvement in morphological parameters of erythrocytes compared to control samples: normocytes content increased 2 times, while the number of echinocytes decreased by 30–40% depending on the hypothermic storage duration. The ability of CBF and the drug Actovegin to help normalize the erythrocytes shape was observed even after 21 days of these cells' storage in hypothermic conditions.

The content of ATP and 2,3-DFG in erythrocytes. In parallel with the study of erythrocytes morphological changes during hypothermic storage, the effect of CBF and the drug Actovegin on the content of phosphorus-containing compounds in erythrocytes was studied. The results of the study showed that the content of ATP in the control erythrocytes samples gradually and significantly decreased. In general, during the entire storage period, the initial value decreased by 2.3 times. Incubation of cells in media containing CBF or the drug Actovegin helped to restore ATP levels to those that were close to baseline. Addition to the incubation medium of the glycolysis inhibitor sodium iodoacetate blocked the stimulating effect of both investigated drugs. As a result, the ATP content remained at the level of control values (Table 1).

Similar changes were observed in the study of the 2,3-DFG content.During the storage of erythrocytes, this indicator significantly decreased and it was only 62% of the initial valueby the final date. After cells incubation in rehabilitation media with CBF or the drug Actovegin, the 2,3-DFG content was restored (Tabl. 2). It should also be noted that the glycolysis inhibitor blocked the 2,3-DFG accumulation.

Criterion of erythrocytes oxygen transport function. At the next stage of the research, the effect of CBF on the erythrocytes oxygen transport function was studied according to standard indicators: oxygen tension (PO_2), carbon dioxide tension (PCO_2) and saturation (SPO_2). The results of these parameters study are given in the Table 3. In the process of erythrocytes hypothermic storage, the O_2tension remained virtually unchanged, but the CO_2 tension increased and saturation decreased to critical values. Addition of CBF to the erythrocytes incubation medium contributed to a probable increase in O_2tension and a decrease in CO_2tension at each of the time control points. Throughout the study period, a pronounced effect of CBF on the saturation index was also recorded.

The ratio of hemoglobin forms. Investigation of the hemoglobin forms ratio in donor blood erythrocytes stored in hypothermic conditions have shown that in the control there is a redistribution between the content of oxy-, deoxy- and methemoglobin in the direction of the share of the latter two increasing. After incubation in rehabilitation medium with CBF, there was an increase in oxyhemoglobin and a parallel decrease in deoxy- and methemoglobin, which resulted in the restoration of the relationship between these indicators at each of the studied storage periods (Table 4).

A similar regularity was found when adding the drug Actovegin to the incubation medium. Thus, erythrocytes incubation in rehabilitation medium with the drug Actovegin contributed to a significant increase in oxyhemoglobin content at each of the observation points. The content of deoxy- and methemoglobin was significantly reduced, which contributed to the O_2Hb: DeoxyHb: MetHb ratio normalization.

Fig. 2 shows the change in the oxygenation coefficient, which is the ratio of the deoxyhemoglobin amount to the oxyhemoglobin

Table 1. **The effect of the human cord blood low-molecular fraction and the drug Actovegin on the ATP content in donor human erythrocytes, μmol/ml of erythromass**

Period of storage, days	Control	CBF	Actovegin	CBF + Ia	Actovegin + Ia
1	5.38 ± 0.07	–	–	–	–
7	3.3 ± 0.32*	5.3 ± 0.29^{+}	5.31 ± 0.44^{+}	3.5 ± 0.16**	3.48 ± 0.11**
14	2.98 ± 0.19*	4.9 ± 0.23*$^{+}$	4.93 ± 0.28*$^{+}$	3.02 ± 0.31**	3.09 ± 0.34**
21	2.3 ± 0.31*	4.05 ± 0.51*$^{+}$	4.21 ± 0.25*$^{+}$	2.38 ± 0.47**	2.4 ± 0.47**

Note: Ia — sodium iodoacetate; * — differences are significant compared to the control indicator for the 1st day of storage ($P < 0.05$); $^{+}$ — differences are significant compared to the corresponding indicator for control ($P < 0.05$); ** — differences are significant compared to the options for erythrocytes incubation in media containing CBF and the drug Actovegin, respectively ($P < 0.05$); $n = 5$.

Table 2. **The effect of the human cord blood low-molecular fraction and the drug Actovegin on the 2,3-DFG content in human donor erythrocytes, μmol/ml of erythromass**

Period of storage, days	Control	CBF	Actovegin	CBF + Ia	Actovegin + Ia
1	5.61 ± 0.18	–	–	–	–
7	4.8 ± 0.32*	5.4 ± 0.09^{+}	5.5 ± 0.13^{+}	4.9 ± 0.11**	5.05 ± 0.18**
14	4.05 ± 0.35*	4.9 ± 0.13*$^{+}$	5.06 ± 0.22^{+}	4.11 ± 0.17**	4.17 ± 0.28**
21	3.5 ± 0.41*	4.9 ± 0.34*$^{+}$	5.36 ± 0.4^{+}	3.58 ± 0.38**	3.77 ± 0.21**

Note: Ia — sodium iodoacetate. * — differences are significant compared to the control indicator for the 1st day of storage ($P < 0.05$); $^{+}$ — differences are significant compared to the corresponding indicator for control ($P < 0.05$); ** — differences are significant compared to the options for erythrocytes incubation in media containing CBF and the drug Actovegin, respectively ($P < 0.05$); $n = 5$.

Table 3. **Change in the parameters of erythrocytes oxygen transport function depending on the storage period and the presence in the incubation medium of the human cord blood low-molecular fraction**

Period of storage, days	Criterion of oxygen transport function					
	PO_2		PCO_2		SPO_2, %	
	Control	CBF	Control	CBF	Control	CBF
1	32 ± 2.0	45.5 ± 1.5*	82.45 ±0.65	77.05 ±1.55*	46 ± 1.1	63.05 ±0,75*
7	29 ± 1.0	51.5 ± 0.5*	96.6 ±1.3	87 ± 1.0*	43.7±1.5	60.3 ±0.4*
14	41 ± 1.0	77 ± 3.0*	111 ±0.3	104.5±1.25*	40.2 ±0.3	58.1 ±0.4*
21	35 ± 2.0	72 ± 1.0*	116 ±1.5	105.2±1.3*	37.9±0.65	55.9±0.5*

Note: * — differences are significant compared to the corresponding control indicator ($P < 0.05$); $n = 6$.

Table 4. **The effect of the human cord blood low-molecular fraction and the drug Actovegin on the hemoglobin forms ratio**

Period of storage, days	Incubationmedium	O_2Hb, %	DeoxyHb, %	MetHb, %
1	Control	73.2 ± 1.32	25.8 ± 2.18	1.1 ± 0.14
7	Control CBF Actovegin	66.3 ± 2.23 73.0 ± 1.9* 79.1 ± 3.07*	27.3 ± 2.44 22.1 ± 2.94* 19.8 ± 0.58*	6.4 ± 0.43 4.9 ± 0.66* 1.1 ± 0.72*
14	Control CBF Actovegin	64.6 ± 3.67 73.9 ± 2.87* 72.3 ± 2.18*	30.0 ± 2.98 23.0 ± 2.48* 21.7 ± 2.09*	7.75 ± 0.54 3.1 ± 0.47* 6.0 ± 0.88*
21	Control CBF Actovegin	60.4 ± 2.49 69.8 ± 2.38* 71.7 ± 2.9*	31.1 ± 1.77 25.8 ± 2.15* 21.9 ± 1.35*	8.5 ± 0.5 4.5 ± 0.08* 6.4 ± 1.13*

Note: * — differences are significant compared to the corresponding control indicator ($P < 0.05$); $n = 6$.

amount. In control erythrocytes samples there was a gradual decrease in the oxygenation coefficient throughout the observation period and on the 21st day of hypothermic storage the indicator decreased by 1.5 times (Fig. 2). CBF increased the oxygenation coefficient after 7 days of erythrocytes hypothermic storage by 37.5%, after 14 days — by 52%, and after 21 days — by 42%. Stimulating effect on the oxygenation coefficient was observed also when adding the drug Actovegin to the incubation medium. Thus, after 7, 14 and 21 days of storage, cell rehabilitation with the drug Actovegin led to an increase in the studied coefficient by 67%, 57% and 73%, respectively (Fig. 2).

Under physiological conditions, human erythrocytes have the shape of a biconcave disk. The shape of erythrocytes has a high sensitivity to changes in medium conditions and composition. So, for many years it is considered a parameter that reflects the normal state of cells [20]. It is known that under conditions of erythrocytes storage at 4–6 °C there is a change in their shape from discocytes (normocytes) to echinocytes, and then to spherocytes. Such deformation is irreversible even after transfusion [21].

In our studies, the shape of erythrocytes that were subjected to hypothermic storage was restored by adding to their incubation medium of CBF or the drug Actovegin at a final concentration of 0.6 mg/ml. Discussing the possible mechanisms of the detected rehabilitative effect of CBF and the drug Actovegin on the erythrocytes morphology, we could make the following assumptions. First, the erythrocytes shape restoration may be associated with an improvement in the cells energy potential as a result of the glycolysis process stimulation and a corresponding increase in phosphorus-containing metabolites in erythrocytes. A similar mechanism was found for the CBF effect on energy metabolism in leukocytes of donated blood [10, 12, 13]. In turn, increasing the ATP level enables to maintain the cytoskeleton structure by regulating the protein kinases activity that phosphorylate actin, spectrin and ankyrin, thereby maintaining the discoid form of erythrocytes [22, 23]. Secondly, this phenomenon may be associated with the direct action of the components contained in the CBF on the erythrocytes' cytoskeleton. This assumption is based on our experiments on the effect of fraction on donor blood leukocytes, using cytocholasin B, which is known to inhibit the cytoskeleton and inhibit glucose transport in cells by blocking glucose transporters of the Glut family [24, 25].

The first assumption is confirmed by studying the ATP and 2,3-DFG content in

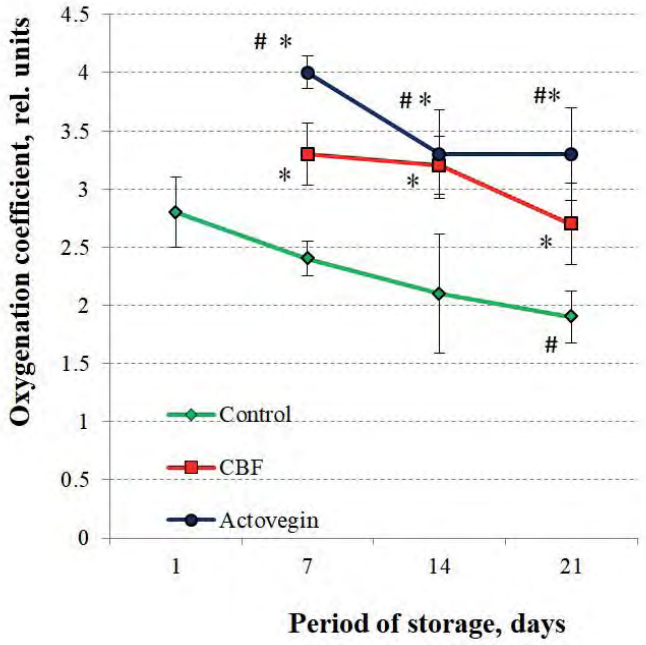

Fig. 2. **The effect of the human cord blood low-molecular fractionand the drug Actovegin on the oxygenation coefficient**

* — differences are significant in comparison with the corresponding control ($P < 0.05$); # — the difference is significant in comparison with the control for the 1st day of storage ($P < 0.05$)

erythrocytes. It has been found that CBF and the drug Actovegin contribute to the increase the content of these compounds in erythrocytes. It is important to note that the glycolysis inhibitor sodium iodoacetate blocks the stimulating effect of both drugs. The obtained data of inhibitory analysis indicate that with the help of the investigated drugs it is possible to influence the intensity of glycolysis reactions and thus stimulate the ATP synthesis and 2,3-DFG formation, as the latter is formed in glycolysis from 1,3-diphosphoglycerate.

As a result of the conducted experiments it has been also found that by CBF adding to the erythrocytes incubation medium it is possible to increase the indicators of their oxygen transport function. Studies of hemoglobin affinity for oxygen in donor blood erythrocytes stored in hypothermic conditions showed that in the control this indicator decreases by 30% for 21 days, and after incubation in a rehabilitation medium with CBF, it increases by 30–50% depending on storage period. This effect can be compared with the stimulating effect of CBF with a molecular weight of up to 5 kDa, found in our previous research [26].

In addition, CBF and the drug Actovegin have been shown to affect the ratio of erythrocytes' hemoglobin forms. It is known that under physiological conditions, hemoglobin in erythrocytes can be in one of three forms — deoxyhemoglobin, methemoglobin or oxyhemoglobin [27].

In our experiments, incubation of erythrocytes stored under hypothermic conditions in the medium with CBF or the drug Actovegin was able to increase significantly the oxyhemoglobin proportion. The CBF and the comparison drug Actovegin had the same effect on the ratio of hemoglobin forms (Table 4, Fig. 2). In total, the obtained data indicate the normalization of erythrocytes oxygen transport function, as deoxyhemoglobin does not contain oxygen molecules, and methemoglobin is not able to perform the function of an oxygen carrier. Based on these data, it can be concluded that both rehabilitation media increase blood oxygen capacity.

The revealed facts can be explained by several reasons. First, it has been found that CBF and the drug Actovegin increase the ATP and 2,3-DFG content (Tables 1 and 2), which regulate the hemoglobin conformation and the reaction of oxygen attachment to it [28]. Secondly, it is possible that the studied drugs contain components that are able to change the hemoglobin conformation independently or as a result of a combined complex action towards its affinity increasing for oxygen.

Conclusions

As a result of the research it was found by the first time that the CBF (5-10 kDa) or the drug Actovegin presence in the incubation medium promoted to restore the morpho-functional characteristics of erythrocytes after hypothermic storage at 2–4 °C for 21 days, which is expressed in a probable increase in normocytes and restoring indicators of their functional state, namely oxygen tension, saturation, ATP and 2,3-DFG and normalization of hemoglobin different forms ratio.

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The authors declare that any pharmaceutical or other companies did not influence the treatment conditions, study design, data collection and analysis, preparation of manuscript, and choice of journal.

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ВІДНОВЛЕННЯ СТРУКТУРНО-ФУНКЦІОНАЛЬНОГО СТАНУ ЕРИТРОЦИТІВ ПІСЛЯ ГІПОТЕРМІЧНОГО ЗБЕРІГАННЯ ЗА ДОПОМОГОЮ НИЗЬКОМОЛЕКУЛЯРНОЇ ФРАКЦІЇ КОРДОВОЇ КРОВІ ЛЮДИНИ І ПРЕПАРАТУ АКТОВЕГІН

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Однією із задач сучасної трансфузіології є збереження властивостей еритроцитів після гіпотермічного зберігання. Від їхнього функціонального стану залежить якість та ефективність трансфузійного середовища. Досліджено можливість використання як субстанції для реабілітувального середовища низькомолекулярної фракції кордової крові людини (ФКК) і препарату Актовегін.

Мета. Дослідити вплив ФКК і препарату Актовегін на морфологію, енергетичний баланс і киснево-транспортну функцію еритроцитів.

Матеріали та методи. Еритроцитарну масу зберігали за умов гіпотермії впродовж 7–21 доби. Кожні 7 діб до зразків додавали ФКК або препарат Актовегін (кінцева концентрація 0.6 мг/мл) та інкубували 1 год при 37 °С. ФКК отримували з кордової крові людини методом ультрафільтрації. Морфологію еритроцитів оцінювали за допомогою світлової мікроскопії. Вміст АТФ та 2,3-ДФГ визначали фотоелектрокалориметричним методом. Сатурацію, напруженість O_2 і CO_2 оцінювали за допомогою аналізатора газів крові. Співвідношення форм гемоглобіну досліджували фотометрично.

Результати. Показано, що ФКК або препарат Актовегін сприяють відновленню морфофункціональних характеристик еритроцитів після 21 доби зберігання при 2-4 °С. Це виражалось у збільшенні кількості нормоцитів і відновленні показників напруженості O_2, сатурації, вмісту АТФ і 2,3-ДФГ та нормалізації співвідношення форм гемоглобіну. Механізм дії цих препаратів пов'язаний з активацією синтезу АТФ та утворенням 2,3-ДФГ.

Висновки. Використання середовищ з ФКК або препаратом Актовегін, дозволило ефективно відновлювати властивості еритроцитів після тривалого зберігання.

Ключові слова: кордова кров, Актовегін, еритроцити, гіпотермія, відновлююче середовище.