UDC: 611-018.5.013.8:615.014.41

THE GENERATION OF REACTIVE OXYGEN SPECIES BY CORD BLOOD NUCLEATED CELLS DURING CRYOPRESERVATION

V. V. Ryazantsev
L. A. Babijchuk
O. O. Mykhailova
P. M. Zubov

Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkiv

E-mail: mixolya@mail.ru

Received 04.10.2014

The generation of reactive oxygen species in different populations of nucleated cells during cord blood cryopreservation was studied. Using cytometry with the 2′,7′-dichlorodihydrofluorescein diacetate dye we assessed the integral content of reactive oxygen species in various populations (lymphocytes, monocytes, granulocytes) of cord blood nucleated cells, depending on the method of isolation, treatment with cryoprotectant and freezing. Furthermore the activity of antioxidant enzymes prior to and after cryopreservation while the cells were loaded with exogenous reactive oxygen species, which source was hydrogen peroxide, was assessed. The results obtained showed that the isolation and cryopreservation of cord blood nucleated cells-according to proposed method, which included the isolation of cells in polyglucinum and freezing with 5% DMSO did not result in a significant increase in level of intracellular reactive oxygen species. In addition the activity of antioxidant enzymes to exogenous hydrogen peroxide inactivation in cryopreserved cell was maintained at the level close to that in the native cord blood. Among all the populations of cord blood nucleated cells the granulocytes were the least resistant to cryopreservation effects.

Key words: nucleated cells, cord blood, cryopreservation, reactive oxygen species.

Cryopreservation of cord blood (CB) nucleated cells (NCs) can cause the changes of their structural and functional properties [1]. This may disorder many biochemical processes, including the cell power supply system, with the following generation of the toxic reactive oxygen species (ROSs). Along with ROS generation during cell vital activity at all the stages of cryopreservation, particularly NCs isolation, treatment with cryoprotectant and freeze-thawing, the reaction of lipid peroxidation (LPO) can be activated, which may potentiate ROSs accumulation and results in the biomolecules' damage [2, 3]. Maintaining the cells integrity during cryopreservation also depends on the functioning of antioxidant enzymes [4].

The purpose was to determine the ROSs level in various populations (lymphocytes, monocytes, granulocytes) of CB NCs, depending on the method of isolation, treatment with cryoprotectant and freeze-thawing, as well as the activity of antioxidant enzymes before and after cryopreservation while the cells were loaded with exogenous ROSs.

Materials and Methods

Object of the research was human cord blood nucleated cells obtained with glucose-citrate solution. Cord blood was obtained from the umbilical vein after mother's informed consent.

The NC concentrates were isolated by several methods: sedimentation in 3% polyglucinum, centrifugation in ficoll-verografin density gradient, [5] and by our own method of two-step centrifugation of the native cord blood with the following obtaining of NC concentrate in autoplasma [6].

The cells were frozen either with 5% DMSO or 10% PEO-1500 (polyethylene oxide) down to −196 °C by specially designed two-stage program with the CryoSON freezer [7]. Cell suspension was supplemented with cryoprotectants at low temperature (2–4 °C), 1:1 (by v/v) [8].

Number of survived NCs was determined by standard method using the cell counter as a ratio of exposed cells in the sample to the amount of intact (before exposure) cells expressed as a percentage.

Phenotyping of nucleated ($CD45^+$)-cells and viability assessment with DNA marker 7-aminoactinomycin D (7AAD) was performed using flow cytometry (FACS Calibur cytometer, Becton Dickinson, USA) and using the BD reagents. To assess $CD45^+$-cells and their viability we used standard protocol for immunophenotyping with CD45FITC and 7AAD. The populations of leukocytes (lymphocytes, monocytes and granulocytes) were determined with dot-plots in coordinates of the side scatter (SSC) and the first channel of fluorescence (FL1: CD45FITC). The cells, which were unstained with 7AAD, were considered as viable. Flow cytometry data were calculated using the CELLQuest Pro software (BD).

ROS production in CB NCs was assessed by flow cytometry with the dye $DCFH_2$-DA, which transforms from non-fluorescent into the highly fluorescent form DCF in the presence of ROSs in a cell [9]. The cells were incubated with 5μM $DCFH_2$-DA in the darkness for 15 min. Activity of antioxidant enzymes was determined after the addition of exogenous H_2O_2 in the cell suspension.

The data are presented as $M \pm m$, the differences significance between the samples were evaluated using Student's t-test with 5% significance level. The sample number included at least 5 experiments.

Results and Discussion

Previously it was shown that the different populations of NCs possesed different cryosensitivity, for example, after cryopreservation with 5% DMSO the highest number of non-viable cells was observed in the fraction of granulocytes [9, 10].

It was topical to investigate ROS production in different populations of NCs under cryoprotectants treatment and freezing conditions. For this purpose the fluorescence of DCF, which was directly-proportioned to the intracellular ROS amount, was measured [11, 12]. For the results analysis the high and middle fluorescence intensity regions were taken into account (Fig. 1, *A*).

Direct DCF fluorescence showed that the content of ROSs after the NCs isolation and their treatment with cryoprotectants significantly differed from that in the cells isolated with Ficoll (Fig. 1, *B*), where we saw a tendency to increasing in ROS level; wherein almost all the fluorescent cells was in high intensity region.

Later we analyzed the relative change in the number of DCF-labeled NCs in regions of high and middle fluorescence intensity, taking it as either an increase or decrease in fluorescence before and after exposure to the studied factor. It was found that the degree of changes in the number of DCF-labeled cells directly depended on the intensity of effect: the greater changes in the relative number of DCF-labeled cells corresponded to the greater increase in antioxidant enzymes activity and ROS generation in these cells. Fig. 2 shows that the NCs treatment with cryoprotectants, irrespectively to the nature of their impact on cells, resulted in no changes in the ROS generation in cells, that corresponded to an insignificant change in relative DCF fluorescence in the studied cells [up to 8% in areas of high (High) and middle (Mid) DCF fluorescence intensity].

A similar pattern was noted for different NCs populations (lymphocytes, monocytes and granulocytes): treatment with cryoprotectants did not cause significant changes in relative DCF fluorescence (Fig. 3).

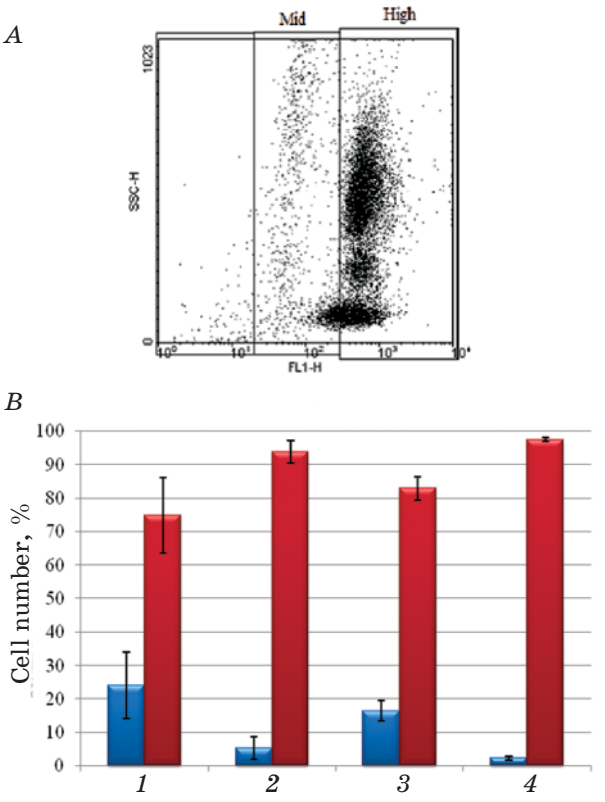

Fig. 1. **Data of standard experiment (*A*) and direct DCF fluorescence of CB NCs (*B*) in regions of high (High) and middle (Mid) fluorescence intensity depending on isolation method and cryoprotectant type used for cells' treatment:**
treatment with 5% DMSO of cells isolated either in polyglucinum (*1*) or Ficoll density gradient (*2*); treatment with 10% PEO-1500 of cells isolated by either two step centrifugation (*3*) or in Ficoll density gradient (*4*)

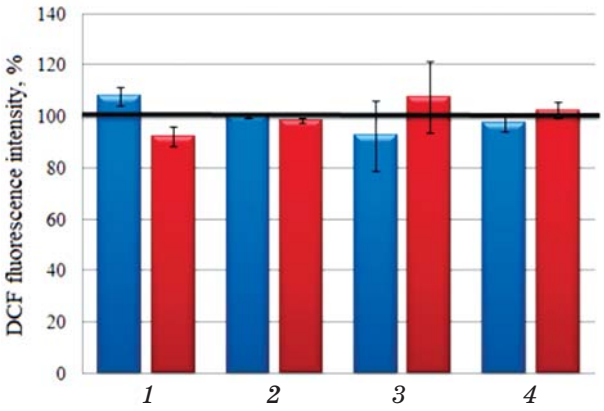

Fig. 2. **Relative change in number of DCF-labeled NCs in regions of high ( ) and middle ( ) fluorescence intensity depending on isolation method and cryoprotectant type used for cells' treatment:** here and in Fig. 3: treatment with 5% DMSO of cells isolated either in polyglucinum (*1*) or Ficoll density gradient (*2*); treatment with 10% PEO-1500 of cells isolated by either two step centrifugation (*3*) or in Ficoll density gradient (*4*)

Further the ROS production by NCs after freeze-thawing (Fig. 4) was investigated. Of importance was the fact that DCF fluorescence intensity in the cells was largely related to their viability. Thus, using most effective methods for isolation and cryopreservation we showed that the cells number in the intense light zone remains high, in contrast to the case when the cells were frozen after isolation by Ficoll were observed the redistribution of DCF-labeled cells with decrease of cell number in the High zone and increase in the Mid zone.

Analysis of DCF relative fluorescence of CD45-labeled NCs showed that the more effective method of cryopreservation, the less pronounced divergence from baseline fluorescence (Fig. 5). It should be noted that in total NCs after thawing were characterized by an increased number of cells with a decreased fluorescence intensity in the Mid zone.

Further we determined the ROS generation in NC populations after freeze-thawing taking into account the heterogeneity of $CD45^+$ fraction, including lymphocytes, monocytes and granulocytes (Fig. 6).

The data showed that most effective method of lymphocytes cryopreservation includes their the isolation by dextran and freezing with DMSO, while the isolation with Ficoll and freezing with DMSO as well as freezing with dextran without cryoprotectant were the least effective. Monocyte population was characterized by minimum changes in the ROS generation during freezing with DMSO regard-

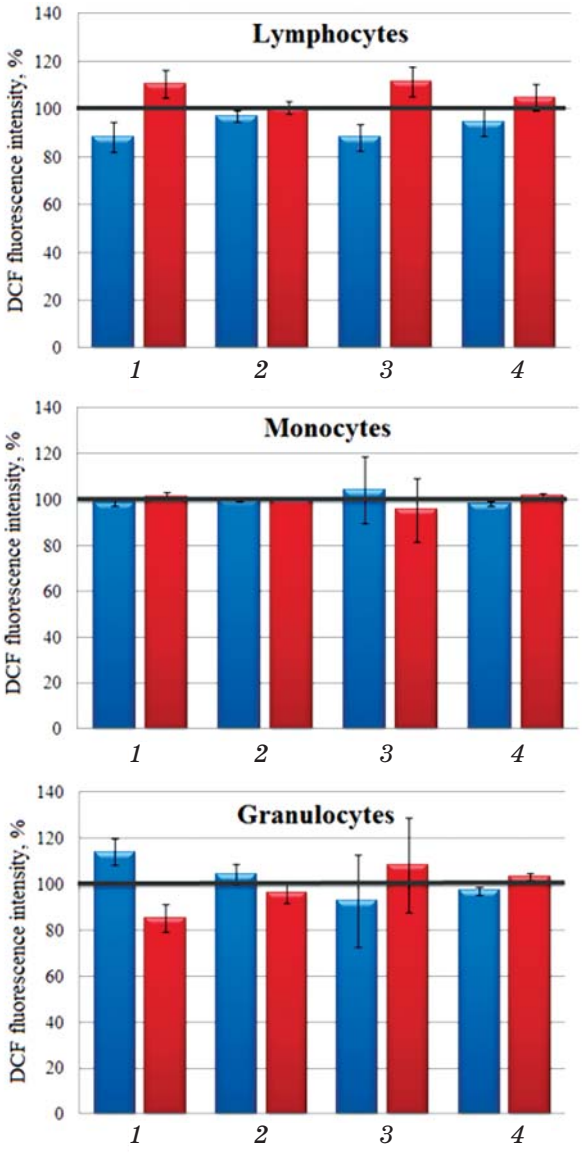

Fig. 3. **Relative change in number of DCF-labeled lymphocytes, monocytes and granulocytes in regions of high ( ) and middle ( ) fluorescence intensity depending on isolation method and cryoprotectant type used for cells' treatment**

less of the isolation methods. In granulocyte population after cryopreservation the greatest changes in the relative fluorescence were observed varying within the range of 36–58%, but were not significant due to their heterogeneity. Of importance was to note that for the granulocyte fraction the intracellular ROS generation activity correlated with their high cryolability.

Further, the activity of NCs antioxidant enzymes was investigated when exogenous hydrogen peroxide was added as inducer of ROS generation. For this purpose we chose the cryopreservation method included NCs isolation

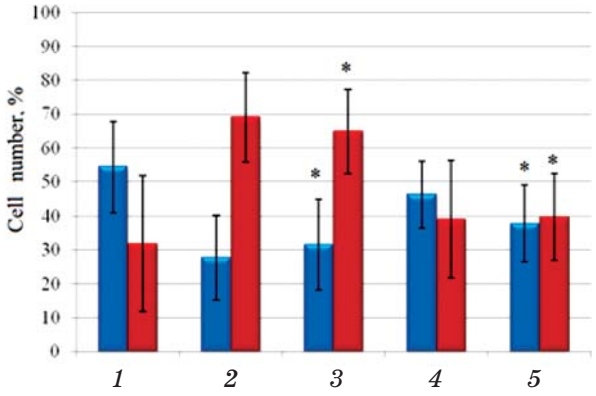

Fig. 4. **Direct DCF fluorescence of CB NCs in regions of high ( ) and middle ( ) fluorescence intensity depending on cryopreservation method:** freezing of the cells isolated in polyglucinum with (*2*) and without (*1*) 5% DMSO; freezing with 5% DMSO of cells isolated in Ficoll (*3*); freezing with 10% PEO-1500 of the cells isolated by either two step centrifugation (*4*) or in Ficoll density gradient (*5*)

* — Here and in *Fig. 5* and *6*:
$P \leq 0.05$ as compared to the value indices before freezing.

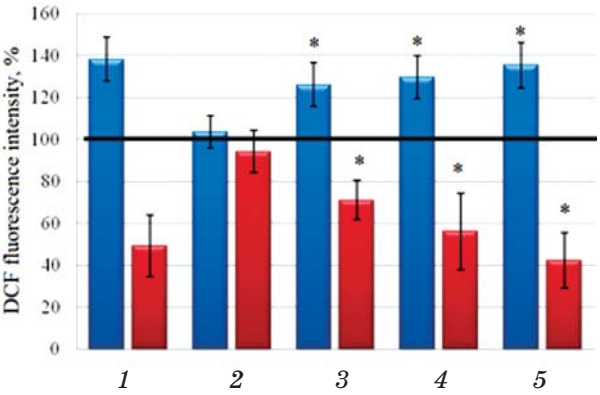

Fig. 5. **Relative change in number of DCF-labeled NCs in regions of high ( ) and middle ( ) fluorescence intensity depending on cryopreservation method:** freezing of the cells isolated in polyglucinum with (*2*) and without (*1*) 5% DMSO; freezing with 5% DMSO of cells isolated in Ficoll (*3*); freezing with 10% PEO-1500 of the cells isolated by either two step centrifugation (*4*) or in Ficoll density gradient (*5*)

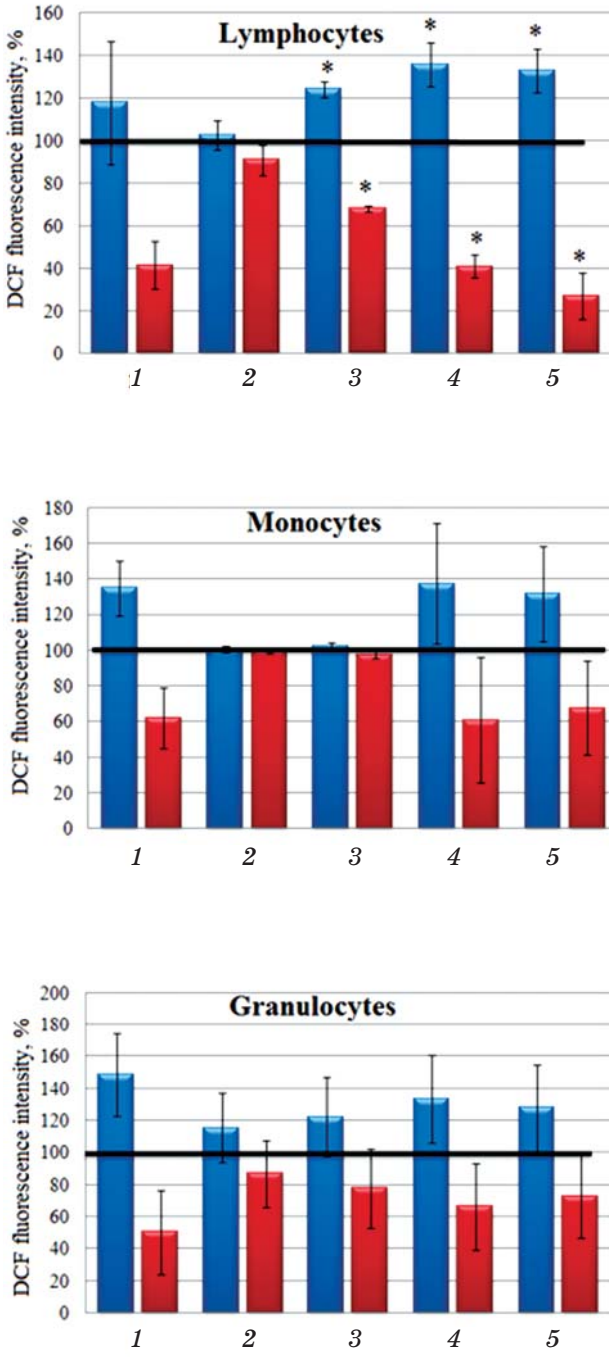

Fig. 6. **Relative change in number of DCF-labeled lymphocytes, monocytes and granulocytes in regions of high ( ) and middle ( ) fluorescence intensity depending on cryopreservation method:** freezing of the cells isolated in polyglucinum with (*2*) and without (*1*) 5% DMSO; freezing with 5% DMSO of cells isolated in Ficoll (*3*); freezing with 10% PEO-1500 of the cells isolated by either two step centrifugation (*4*) or in Ficoll density gradient (*5*)

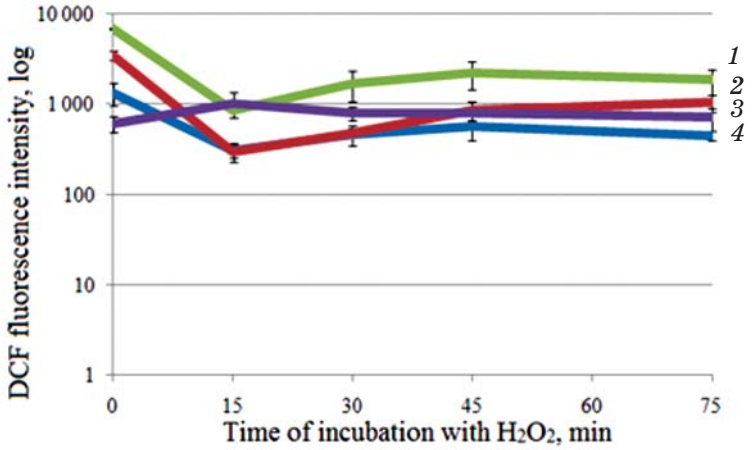

Fig. 7. **DCF fluorescence intensity of CB NCs depending on time of incubation with exogenous H_2O_2:**
1 — whole CB*;*
2 — isolation in polyglucinum;
3 — treatment with 5% DMSO;
4 — freeze-thawing

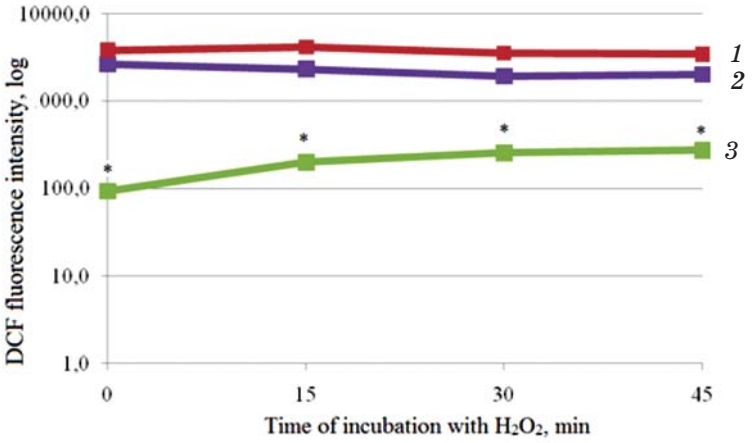

Fig. 8. **DCF fluorescence intensity of lymphocytes (*1*), monocytes (*2*) and granulocytes (*3*) when adding exogenous H_2O_2 after cells isolation with dextran and freezing with 5% DMSO:**
* — $P \leq 0.05$ significant difference of I_{DCF} compared with monocytes and granulocytes

with dextran and freezing with 5% DMSO. It was shown that the addition of exogenous H_2O_2 to NCs both after isolation and treatment with DMSO did not change the time course dynamics of the fluorescence intensity (I_{DCF}) related to that in native CB (Fig. 7).

At the same time for the case of the exogenous H_2O_2 addition to the NCs after freeze-thawing the time course dynamics of I_{DCF} fluorescence was different and characterized by the absence of reduction in I_{DCF} for the first 15 min of incubation with H_2O_2. Before freezing the fluorescence intensity in 15 min of incubation with H_2O_2 significantly decreased compared to the initial level. Obviously this was due to the inactivation of exogenous ROSs, but in the dynamics up to 45 minutes, these differences were smoothed over and decreased to the level typical for the cells in native CB. Similar cell response to the addition of exogenous H_2O_2 suggests the possibility of the antioxidant enzymes activity recovery after cryopreservation using this method.

The main contribution to the I_{DCF} increase after the addition of exogenous H_2O_2 provided by the populations of monocytes and granulo-

cytes (Fig. 8), while the I_{DCF} of lymphocytes remained low, significantly differing from I_{DCF} of monocytes and granulocytes, demonstrating their resistance to exogenous H_2O_2 and high activity of antioxidant enzymes was kept.

Thus, the results obtained showed that the isolation and cryopreservation of different CB NC populations (lymphocytes, monocytes, granulocytes) by the proposed method, which includes cells' isolation with polyglucinum and freezing with 5% DMSO, did not resulted in a significant increase in the amount of intracellular ROSs, moreover the activity of antioxidant enzymes related to the inactivation of exogenous hydrogen peroxide remains at the level close to the activity in native cord blood. Our experiments confirmed the population of granulocytes was the least resistant to the effects of cryopreservation factors.

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УТВОРЕННЯ АКТИВНИХ ФОРМ КИСНЮ ЯДРОВМІСНИМИ КЛІТИНАМИ КОРДОВОЇ КРОВІ ЗА КРІОКОНСЕРВУВАННЯ

В. В. Рязанцев
Л. А. Бабійчук
О. А. Михайлова
П. М. Зубов

Інститут проблем кріобіології і кріомедицини НАН України, Харків

E-mail: mixolya@mail.ru

Вивчено утворення активних форм кисню в різних популяціях ядровмісних клітин кордової крові за кріоконсервування. Методом цитофлуориметрії за допомогою барвника 2′,7′-дихлородигідрофлуоресцеїндіацетату здійснено оцінювання інтегрального вмісту активних форм кисню в ядровмісних клітинах кордової крові різних популяцій (лімфоцити, моноцити, гранулоцити) залежно від способу виділення, оброблення кріопротектором і заморожування, а також активності антиоксидантних ензимів до і після кріоконсервування за умов навантаження клітин екзогенними активними формами кисню, джерелами яких був пероксид водню. На підставі одержаних даних було показано, що виділення і кріоконсервування різних популяцій ядровмісних клітин кордової крові (лімфоцити, моноцити, гранулоцити) із застосуванням запропонованого методу, зокрема після виділення клітин в поліглюкіні й заморожування з 5% ДМСО, не призводить до істотного збільшення кількості внутрішньоклітинних активних форм кисню, при цьому активність антиоксидантних ензимів унаслідок інактивації екзогенного пероксиду водню зберігається на рівні, близькому до активності в цільній кордової крові. Найменш стійкою до дії факторів кріоконсервування є популяція гранулоцитів.

Ключові слова: ядровмісні клітини, кордова кров, кріоконсервування, активні форми кисню.

ОБРАЗОВАНИЕ АКТИВНЫХ ФОРМ КИСЛОРОДА ЯДРОСОДЕРЖАЩИМИ КЛЕТКАМИ КОРДОВОЙ КРОВИ ПРИ КРИОКОНСЕРВИРОВАНИИ

В. В. Рязанцев
Л. А. Бабийчук
О. А. Михайлова
П. М. Зубов

Институт проблем криобиологии и криомедицины НАН Украины, Харьков

E-mail: mixolya@mail.ru

Изучено образование активных форм кислорода в различных популяциях ядросодержащих клеток кордовой крови при криоконсервировании. Методом цитофлуориметрии с помощью красителя 2′,7′-дихлородигидрофлуоресцеиндиацетата осуществлена оценка интегрального содержания активных форм кислорода в ядросодержащих клетках кордовой крови различных популяций (лимфоциты, моноциты, гранулоциты) в зависимости от способа выделения, обработки криопротектором и замораживания, а также активности антиоксидантных энзимов до и после криоконсервирования в условиях нагрузки клеток экзогенными активными формами кислорода, источником которых был пероксид водорода. На основании полученных данных было показано, что выделение и криоконсервирование различных популяций ядросодержащих клеток кордовой крови (лимфоциты, моноциты, гранулоциты) при использовании предложенного метода, в частности после выделения клеток в полиглюкине и замораживания с 5% ДМСО, не приводит к существенному увеличению количества внутриклеточных активных форм кислорода, при этом активность антиоксидантных энзимов вследствие инактивации экзогенного пероксида водорода сохраняется на уровне, близком к активности в цельной кордовой крови. Наименее устойчивой к действию факторов криоконсервирования является популяция гранулоцитов.

Ключевые слова: ядросодержащие клетки, кордовая кровь, криоконсервирование, активные формы кислорода.