ORIGINAL ARTICLES

Evaluation of cryopreserved murine and human hematopoietic stem and progenitor cells designated for transplantation

Assist. Prof. **Bela Balint**[*], MD, PhD, Prof. **Zoran Ivanović**[†], MD, PhD, **Marijana Petakov**[†], MD, PhD, Col. Assist. Prof. **Jovan Taseski**[*], MD, PhD, **Danilo Vojvodić**[‡], MD, MSc, Assoc. Prof. **Gordana Jovčić**[†], MD, PhD, Assoc. Prof. **Diana Bugarski**[†], MD, PhD, Lt. Col. **Slobodan Marjanović**[||], MD, PhD, Prof. **Milomir Malešević**[||], MD, PhD, Prof. **Nevenka Stojanović**[†], BSc, PhD

Military Medical Academy, [*]Institut of Transfusiology, [‡]Institut for Medical Research, [||]Clinic of Hematology, **Belgrade**; [†]**Institut for Medical Research, Belgrade.**

The influence of five different cryopreservation protocols on the quality and/or quantity of frozen cells was investigated on mouse bone marrow cells and human peripheral blood mononuclear cells (MNC). The efficiency of the protocols was evaluated on the basis of the recovery of very primitive pluripotent hematopoietic stem cells (MRA), pluripotent progenitors (CFU–Sd12), committed granulocyte–monocyte progenitors (CFU–GM) of mouse cells after thawing. The recovery of MRA, CFU–Sd12 and CFU–GM varied depending on the type of freezing procedure and cryoprotector (DMSO) concentrations used. It was shown that the controlled–rate protocol was more efficient, enabling better recovery of all categories of progenitor cells in frozen samples. The most efficient was the controlled–rate protocol of the cryopreservation designed to compensate for the release of fusion heat, which enabled the best recovery of CFU–GM (73.0±8.8%) and CFU–Sd12 (90.0±15.9%) when combined with 5% DMSO concentration (protocol 4). On the contrary, a better recovery (79.8±13.5%) of very primitive stem cells (MRA) was achieved only when the higher concentration (10%) DMSO was used in combination with a five-step protocol of cryopreservation (protocol 1). These results pointed out the adequately used controlled-rate freezing to be essential for a highly efficient cryopreservation of some of the categories of hematopoietic stem and progenitor cells. At the same time, it was obvious that a higher DMSO concentration was necessary for the cryopreservation of MRA, but not for more mature progenitor cells (CFU–S, CFU–GM). These results imply the existence of a mechanism that decreases the intracellular concentration of DMSO in MRA cells, which is not the case in less primitive progenitors. For human MNC, the recovery and viability of the cells, as well as the engraftment potential of cryopreserved cells after thawing were investigated. Cryopreservation protocol 1 resulted in better MNC recovery (82.7±10.4%) than protocol 3 (49.9±15.1%). The mean recovery of MNCs (collected from patients for autologous transplantation) was 78.5±7.3% (protocol 1) and 53.1±26.2% (protocol 3). The obtained favorable recovery of thawed cells and rapid reconstitution of hematopoiesis (on the day 11[th] following the transplantation) in patients confirmed that the controlled-rate freezing in combination with optimal DMSO concentration was able to obtain sufficient progenitor cryoprotection.

Key words: **hematopoietic stem cells; cryopreservation; hematopoietic stem cell transplantation; dimethyl sulfoxide; cytological techniques; bone marrow cells; clinical protocols.**

Introduction

Successful performance of autologous transplantation of hematopoietic stem and progenitor cells (HSPCs) requires both efficient collection and cryopreservation procedures for obtaining an acceptable cell yield and recovery (1–5). The aim of cryopreservation protocols used is to minimize cell injuries during the freeze-thaw process (cryoinjuries). Generally, the cell injury may be the result of extensive cellular dehydration ("solution effect") and/or intracellular ice crystallization ("mechanical cell damage") (6–9). These independent mechanisms, the first one expressed primarily during low rate freezing, and the second one during rapid freezing, can act simultaneously leading to cytolysis. Thus, the determination and use of the optimal cooling velocity, specific for each type of isolated cells, should be considered. For a successful HSPC cryopreservation, a cryoprotective agent that decreases osmotic gradient and the vapor pressure difference between the intra- and extracellular area is also needed. Dimethyl sulfoxide (DMSO) is commonly used as a cryoprotectant in different (2.2% to 10%) concentrations (10–20). Finally, a higher degree of cell destruction has been evidenced when the transition period from liquid to solid phase (release of the fusion heat) was prolonged (9, 21, 22). Although the HSPC cryopreservation procedures are already used routinely, some problems related to the optimal cooling velocity during controlled-rate freezing and the choice of the cryoprotective agent at the appropriate concentration are still unresolved.

The purpose of this study was to establish a cryopreservation protocol enabling efficient cell recovery and viability after thawing and improving the clinical effectiveness of the HSPCs used for transplantation. Five different cryopreservation protocols were compared, two of our original controlled-rate and one noncontrolled-rate freezing procedures combined with different concentrations of DMSO. We hypothesed that controlled-rate cryopreservation (cooling rate = 1°C/min) with compensation of released heat of the fusion during the transition period from liquid to solid phase (cooling rate = 2°C/min), combined with higher concentration of DMSO (10%) would result in the best HSPC recovery compared to the other freezing procedures and lower concentration (5%) of the same cryoprotective agent. These protocols were first evaluated on murine bone marrow cells, on the basis of recovery after thawing of committed granulocyte-monocyte progenitors (CFU-GM), pluripotent progenitors (CFU-Sd12 = Colony Forming Unit-Spleen day 12) and very primitive pluripotent stem cells determined by marrow repopulating ability assay (MRA_{CFU-GM}). The cryopreservation protocols were further tested on human peripheral blood mononuclear cells (MNCs), harvested both in steady-state hematopoiesis and following the mobilization. The recovery and viability of MNC, as well as the $CD34^+$ cell quantification after thawing were investigated. In addition, the engraftment potential of cryopreserved cells was evaluated through the reconstitution of hematopoiesis in patients treated with autologous HSPC transplantation.

Methods

Ecxperimental animals. CBA/H-mice of both sexes, weighing 20–22 g, when 12–15 weeks old (Breeding Facilities of the Institute for Medical Research, Military Medical Academy, Belgrade) were used as donors of bone marrow cells, as well as recipients of both unfrozen (control) and frozen bone marrow cells in the CFU-Sd12 and MRA assays. Cells from both femurs were flushed out with Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and subsequently pooled, washed and resuspended in DMEM, as described previously (21-23).

Patients and healthy subjects. The study included 30 healthy persons, both males and females, 19 to 44 years old and twenty patients with leukemia (CML, ALL and ANLL), multiple myeloma, Hodgkin and non-Hodgkin lymphoma and extragonadal nonseminious tumor.

Blood (450 mL) from healthy subjects was collected in steady-state hematopoesis in quadruple blood bag systems (Terumo Corporation, Tokyo, Japan). MNCs were separated from buffy-coat by centrifugation, utilizing Ficoll-Paque gradient agent (Pharmacia Fine Chemicals AB, Uppsala, Sweden) (23, 24). MNCs were resuspended in IMDM medium, and samples were taken into thermo-stable plastic tubes (2 ml per tube) for the *in vitro* testing and freezing according to specific cryopreservation protocols (23, 24).

Apheresis procedures. MNC (with HSPC) harvest was performed with COBE Spectra (Cobe BCT, Lakewood, Colorado, USA) blood cell separator when the number of peripheral blood leukocytes was in the range of 3-5 x 10^9/l. Mobilization of HSPC was achieved with the application of polychemiotherapy protocol and G-CSF (Neupogen, Hoffmann-La Roche Ltd. Switzerland), 10-16 μg/kg of body mass daily. The average volume of blood processed by one apheresis procedure was 14.2±1.5 l and the average volume of cell suspension obtained was 0.18±0.02 l.

Cell quantification methods. The number of nucleated cell (NCs) in the murine bone marrow cell suspensions and humans' MNC was quantified in Spencer's chamber following the dilution in hemocytometer with Türck's solution (22, 23). The viability of the cells was evaluated using the trypan blue exclusion test (22–25).

The number of the CFU-GM was determined using methyl cellulose cell culture assay with lung or leukocyte conditioned medium as a source of colony stimulating activity (CSA) (22).

The CFU-Sd12 number was determined in recipient mice irradiated with 9 Gy X-irradiation (RT 305, Philips, The Netherlands), as described previously (22, 26–30). Immediately after irradiation, the recipient mice were *iv* injected with 4-6x10^4 previously cryopreserved (five different protocols) or unfrozen (control group) bone marrow cells (22). The spleens of these recipients were removed 12 days later (CFU-

-Sd12 assay) and fixed in Telleyesniczky's fluid for the counting of visible splenic colonies (23, 26–30).

MRA was estimated on the basis of the number of the newly generated CFU-GM in the bone marrow of irradiated mice, 14 days after inoculation of the test bone marrow cell suspension. The mice were irradiated as described above for CFU-S assay, and injected with $1.5–5x10^5$ fresh or previously frozen and thawed cells. Fourteen days after the injection, mice (5–7 in each group) were sacrificed, the femurs were flushed with DMEM, and an appropriate number of cells ($5–10x10^4$) was placed in methylcellulose culture mixture. The CFU-GM number was determinated as described above and calculated per one femur of the recipient mice. This number represented the MRA of the test sample inoculated in one mouse. The results were then normalized to $1x10^6$ theoretically injected cells, and these values represented the standard MRA value per $1x10^6$ cells in the particular sample tested. To get the total MRA of the particular sample, the above value was multiplied by the actual number of cells present (expressed in millions) both before and after cryopreservation at the moment of MRA determination (injection of mice) (22). The MRA recovery after cryopreservation was calculated as the ratio (percentage) of the total MRA values of the particular sample before and after cryopreservation.

The number of human peripheral blood MNCs and their viability were determined in the same way as described above for murine bone marrow nucleated cells. For the detection of CD34+ cells, MNCs were first separated utilizing gradient agent and then incubated (30 minutes at 4±2°C) with the monoclonal antibodies of HPCA2 specificity (CD34+) conjugated with fluoroscein isotyocyanate – FITC (Becton Dickinson, Heidelberg, Germany). Upon the staining, the percentage of CD34+ cells was analyzed by flow cytometry using Epics device (Coulter, Krefeld, Germany) (24).

Cryopreservation. For the freezing of murine bone marrow cells, as well as human peripheral mononuclear blood cells, the noncontrolled-rate freezing-procedure and controlled-rate freezing procedures were performed using DMSO (Dimethyl sulfoxide, Serva Feinbiochemica, USA), as a cryoprotective agent, in a final concentration of 5% or 10%, (22–24). Our original five steps procedure (22), named as procedure 1, (protocols 1. and 4.) for controlled-rate freezing consisted of five stages: I = 5°C/min, to 0°C; II = 0°C/min, for 10 minutes (equilibration); III = 2°C/min, for 5 minutes; IV = 1°C/min, for 35 minutes and V = 5°C, for 5 minutes (Fig. 1).

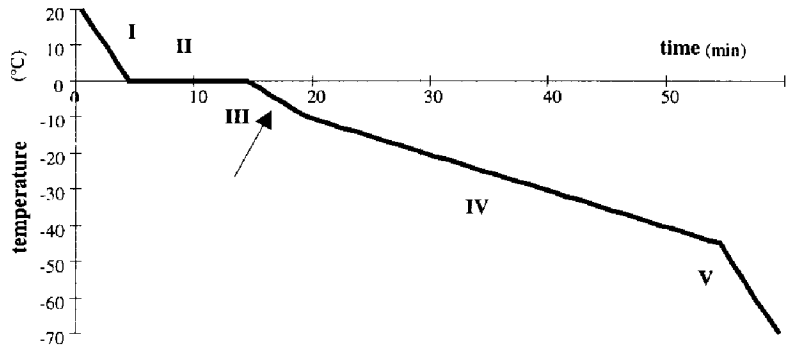

Fig. 1 – Procedure 1 for five-step controlled-rate freezing.
Note step III, corresponding to the release of the fusion heat (arrow).

Four steps procedure – procedure 2, (protocols 2 and 5) consisted of four stages: I = 5°C/min, to 0°C; II = 0°C/min, for 10 minutes (equilibration); III = 1°C/min, for 45 minutes and IV = 5°C, for 5 minutes.

Controlled-rate freezing procedures (protocols 1, 2, 4 and 5) were accomplished by Planer R203/200R (Planer Products Ltd, England) and were performed at the temperature of -70°C for 54 minutes (procedure 1) or for 59 minutes (procedure 2). In all protocols employed, after the completion of freezing, the cell suspensions were placed into a mechanical freezer at a temperature of -90±5°C.

The noncontrolled-rate freezing-procedure (cryopreservation protocol 3) was performed by placing the cell suspension into a mechanical freezer at -90±5°C (22–24) where they remained until thawing.

The cell samples were thawed in a water bath at 37±3°C. The cryoprotective agent present in thawed cell samples was removed by a washing procedure with DMEM, centrifugation for 15 minutes at 600 g, which was followed by cell resuspension in DMEM for murine bone marrow cells or IMDM for human MNC (22, 24).

Cell suspensions intended for the treatment of patients were reinfused through catheter placed in subclavian vein, immediately after thawing, without removing the cryoprotective agent. Patients were premedicated with 40–120 mg of methylprednisolone (Lemod-Solu, Hemofarm, Vršac, Yugoslavia) and 5–10 ml of 10% calcium gluconate solution (Calcii Gluconatis, Pro-Med, Pirot, Yugoslavia). Stimulated diuresis was achieved by the infusion of 2500 ml saline and the application of 20–40 mg of furosemid (Lasix, Jugoremedija, Zrenjanin, Yugoslavia) (24).

Reconstitution of hematopoiesis. The efficacy of the engraftment, i.e., the reconstitution of hematopoiesis in patients treated with autologous transplantation of HSPC was

evaluated according to the established criteria for hematologic recovery – leukocyte count ≥ $1x10^9$/L, neutrophil count ≥ $0.5x10^9$/L and platelet count ≥ $30x10^9$/L for at least three successive days (2, 4, 23, 24).

Statistical analysis. The results were expressed as a mean value ± standard deviation (SD). Statistical analysis of the tested parameters before and after cryopreservation was performed by the ANOVA-test (Statgraf 4.0).

Results

The results obtained for unfrozen murine bone marrow NC count and viability were: N_{NC} = 3.9±0.56x10^6/mL; VB_{NC} = 93.3±5.9%. The numbers of the unfrozen control CFU-GM and CFU-Sd12 were CFU-$GM_{(per\ 5x10^4\ cells)}$ = 103.5±15.7 and CFU-$Sd12_{(per\ 10^5\ cells)}$ = 103.5±15.7

Subsequently after thawing and washing procedures, the recovery and viability of NC were determined. As shown in Figure 2., similar recovery of NC was obtained by all employed cryopreservation protocols, ranging from 70.3±17.1% for protocol 5 to 87.9±6.6% for protocol 4.

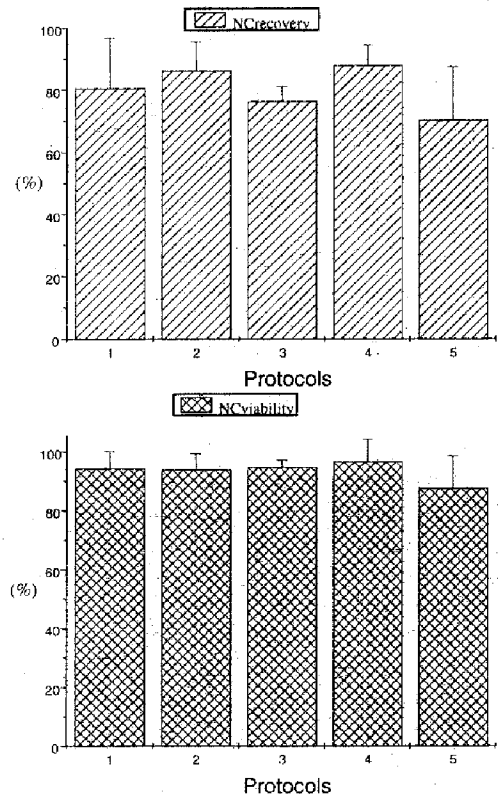

Fig. 2 – The recovery and viability of murine bone marrow NCs after cryopreservation

The results are mean ± SD; The data are pooled from three experiments.

$NC_{RECOVERY}$ = cryopreserved/unfrozen (control group) nucleated cell count.

NC_{VB} = nucleated cell viability (determined immediately after thawing and washing of the cells).

There were no statistical differences in the recovery of cryopreserved NC neither in comparison to the control group NCs, nor between cells frozen by different protocols. Regardless of the cryopreservation procedures used, controlled-rate protocols 1, 2, 4 and 5 or noncontrolled-rate protocol 3, high viability of NC after thawing was determined, and it ranked from 87.6±10.8% to 96.5±7.7%.

The determination of CFU-GM and CFU-Sd12 recovery in the thawed samples of cryopreserved bone marrow cells revealed that the highest recovery of these progenitor cells was achieved when protocol 4 was used, i.e. a five-step controlled-rate freezing procedure and 5% DMSO (73.0±8.8% for CFU-GM and 90.0±15.9% for CFU-Sd12) (Fig. 3).

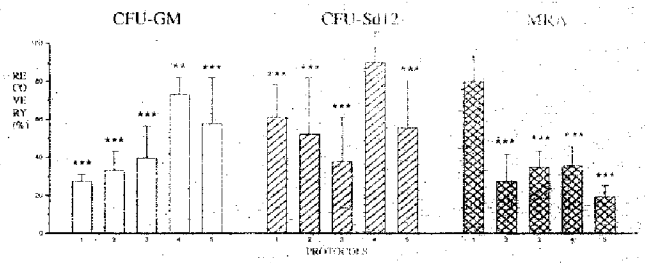

Fig. 3 – The recovery of murine bone marrow progenitor cells (CFU-GM, CFU-Sd12 and MRA) after cryopreservation.

Results are mean ± SD from three separate experiments.

Significant difference cryopreserved cells vs. control:

$^{**}p < 0.01$; $^{***}p < 0.001$.

However, although no differences in the recovery and viability of NC were observed following cryopreservation by different protocols, high variations in the recovery of CFU-GM and CFU-Sd12 progenitor cells were noticed, for CFU-GM ranging from 27.5±3.6% to 73.0±8.8% and for CFU-Sd12 from 37.8±23.0% to 90.0±15.9%.

The efficiency evaluation of the cryopreservation protocols used for CFU-GM and CFU-Sd12 progenitor cells pointed out the controlled-rate freezing procedure to be superior. Namely, the use of five-step controlled-rate freezing procedure with 5% DMSO (protocol 4) did not cause reduction in the number of the CFU-Sd12 in the thawed bone marrow cells *vs.* the control group, while on the other hand, noncontrolled-rate freezing procedure (protocol 3) resulted in a significant reduction of the CFU-Sd12 number *vs.* control group. Highly significant differences were also noticed when the four- and five-step controlled-rate freezing procedures (protocols 2 and 4) were compared, in favor of protocol 4 for both CFU-GM and CFU-Sd12 recovery. Furthermore, significant differences were found between protocols employing five-step controlled-rate freezing procedure but with different DMSO concentrations, 10% DMSO (protocol 1) and 5%

DMSO (protocol 4), again for both the recovery of CFU-GM ($p<0.01$) and CFU-Sd12 ($p<0.05$) indicating that the viability of MNC cryopreserved by these two protocols was similar (Table 1).

Table 1

The recovery and viability of human MNC after cryopreservation
(cells collected in steady state hematopoiesis)

	Recovery [%]	Viability [%]
Before freezing	/	96.8±1.7
Protocol 1	82.7±10.4	87.9±6.1
Protocol 3	49.9±15.1*	79.2±6.7

Protocol 1 = control-rate freezing procedure (with compensation of released heat of fusion) and 10% DMSO;
Protocol 3 = uncontrolled-rate freezing procedure.
* significant difference cryopreserved cells vs. control and protocol 3 vs. Protocol 1 ($p<0.01$).

the optimal DMSO concentration for these progenitor cells was 5% DMSO.

The results obtained for MRA cell recovery after the use of five different cryopreservation protocols (Fig. 3) have shown that efficient cryopreservation of these cells could be achieved only with the appropriate combination of DMSO concentration and controlled-rate freezing, taking into consideration the release of the fusion heat, i.e., five-step controlled-rate freezing with higher DMSO concentration (10%) – protocol 1. When 5% concentration of DMSO was used, the recovery of MRA cells was app. 60-80% lower regardless of the freezing procedure, thus pointing to the importance of DMSO concentration.

According to the results obtained for murine bone marrow cells, the differences in the recovery of human MNC were compared only between protocols 1 and 3, i.e., five-step controlled-rate freezing and non-controlled freezing procedures. The results obtained for MNCs harvested from the healthy subjects demonstrated significantly higher recovery of the MNC cryopreserved by protocol 1, while

The suspensions of MNC (with HSPC) were also obtained from five patients with leukemia. MNC were harvested by apheresis after mobilization and were then used for autologous peripheral blood stem cell transplantation after cryopreservation by protocol 1. However, prior to the transplantation, 2 ml samples of these cells were taken for *in vitro* testing of the efficacy of cryopreservation protocols 1 and 3. The results of the cell count and viability before and after cryopreservation are presented in Table 2.

Table 2

The recovery and viability of human MNC after cryopreservation
(cells collected after mobilization)

	Recovery and viability					
	AphP I	AphP II	AphP III	AphP IV	AphP V	Total
N_1 [%]	81.3	71	74.8	75.5	89.7	78.5±7.3
N_3 [%]	29.0	80.7	82.5	35.0	38.1	53.1±26.2
VB_K [%]	99.5	97.4	98.8	97.5	96.4	97.9±1.2
VB_1 [%]	87.5	93.7	65.7	55.2	83.2	77.1±16.1
VB_3 [%]	42.0	86.0	68.1	41.0	43.0	47.6±32.5

AphP = apheresis product;
$N_{1;3}$ = recovery of MNC, cryopreserved by protocols 1 and 3;
VB_K = viability of MNC before freezing;
$VB_{1;3}$ = viability of MNC, cryopreserved by protocols 1 and 3.

Although it was apparent that the recovery of MNC cryopreserved by protocol 1, controlled-rate freezing, was much higher (78.5±7.3%) compared to the recovery obtained by protocol 3, noncontrolled-rate freezing (53.1±26.2%), the differences were no statistically significant due to significant intergroup variations in the results. The analysis of the MNC viability revealed significantly better values obtained by controlled-rate freezing than by noncontrolled-rate freezing procedure.

The suspensions of MNC for autologous peripheral blood stem cell transplantation were also obtained from the other 15 patients and cryopreserved by protocol 1. The ave-

rage yield was 2.1-6.5x10^8/kg (5.1±1.8x10^8/kg) body mass of the patient for MNC and 2.3-34.5x10^6/kg (6.53±2.2x10^6/kg) for CD34$^+$ cells. After thawing, the recovery of MNC was 93.4% and their viability 90.3%. These patients were treated with rhG-CSF subcutaneously in daily doses of 5 μg/kg body mass after the transplantation and the reconstitution of hematopoiesis was achieved after 11 days in average.

Discussion

The influence of different cryopreservation procedures and protocols on the quality and/or quantity of frozen cells was investigated on murine bone marrow cells and human peripheral blood mononuclear cells. The usage of murine bone marrow cells allows these studies on different stages of hemopoietic cells including the most primitive subpopulations. In the experiments presented here significantly better recovery of bone marrow progenitors CFU-GM, CFU-Sd12 and MRA cells was observed after the application of the controlled-rate cryopreservation procedure compared to the noncontrolled-rate procedure. The observed differences were not related to the changes in the number or viability of nucleated bone marrow cells. When the controlled-rate freezing procedures were compared, the best recovery of CFU-GM and CFU-Sd12 was achieved with the Protocol 4. The difference between this protocol and the other controlled-rate protocols (1, 2 and 5) was that in Protocol 4 the lowest concentration of DMSO (5%) was combined with the five-step freezing procedure. However, when the same five-step freezing procedure was performed in the presence of 10% DMSO (protocol 1), an evidently lower recovery of both CFU-GM and CFU-Sd12 was obtained after thawing the bone marrow cells.

Results obtained for the recovery of MRA cells (pre-CFU-S) were slightly different. Namely, a very good survival of pre-CFU-S was achieved only with the five-step protocol in combination with 10% DMSO concentration. Lower concentration of DMSO (5%) had no protective effect even if it was used in combination with the five-step freezing procedure. These results imply that for the preservation of MRA cell population and their functional capability both five-step controlled-rate cryopreservation procedure and higher DMSO concentration are optimal. The possible reason for the better MRA recovery using higher DMSO concentration may be related to the fact that a higher extracellular DMSO concentration is required in order to achieve and maintain a sufficient intracellular concentration of DMSO, necessary for the cryopreservation of pre-CFU-S. It can be assumed that some membrane mechanism, such as P-glycoprotein transport system, which is known to be present in very primitive hematopoietic stem cells, could eliminate intracellular DMSO from these cells by active transport (31). This membrane mechanism is also present with a very high frequency in leukemic stem and progenitor cells (32) and thus, it could be a cause of the greater sensitivity of these cells to cryopreservation (32–34). Further studies are needed to investigate new modalities for the elimination of leukemic cells from autografts by freezing/thawing procedures.

For human HSPC, although numerous cryopreservation protocols are currently used in clinical practice (2, 22, 35), the optimal procedure is not defined yet. In this study, optimal conditions for the cryopreservation of human peripheral blood MNC (with HSPC) harvested by aphersesis in both in steady-state hematopoiesis and after mobilization were investigated. It is well known that peripheral blood HSPC cryopreservation procedure must be adapted to the conditions that depend on the following: (a) higher cell count in the apheresis product (particularly after the mobilization) compared to the bone marrow aspirate; (b) higher prevalence of the mature blood cells; (c) presence of plasma proteins in the product, and (d) absence of lipid and bone tissue particles in the apheresis product (2, 22–24). Since in apheresis product mature blood cells predominate, in the course of cryopreservation their protection should be secured, or their number decreased prior to freezing. Otherwise, cytolysis and consequential release of intracellular substances will result in cell clumping during and/or after thawing. DMSO has been proved to be an efficient cryoprotective agent not only for HSPC, but also for the platelets and granulocytes (16, 23). However, DMSO does not protect erythrocytes during cryopreservation, so that erythrocyte count in the apheresis product should be as low as possible (22–24). In our study, the erythrocytes numbers were very low (hematocrit less than 0.05 in average) and there was no need for their removal from MNC prior to freezing.

According to the obtained results for murine bone marrow hematopoietic cells, the differences in the recovery of human MNC were compared only between protocols 1 and 3, i.e., five-step controlled-rate freezing combined with 10% DMSO and non-controlled freezing procedures. The results obtained for the recovery of MNC harvested from healthy subjects, as well as from five patients with leukemia demonstrated that using protocol 1 there was no statistically significant decrease in their count after the thawing compared to the controls. On the contrary, noncontrolled-rate freezing (protocol 3) resulted in reduced recovery of MNC both *vs.* the controls and cells cryopreserved by protocol 1, thus, confirming the superiority of protocol 1. When the viability of thawed MNC was analyzed significant reduction in comparison to the controls was observed, regardless whether the controlled- or noncontrolled-rate freezing was used. However, when these two protocols were compared significantly lower viability was obtained by non-controlled-rate freezing. Although there are some reports (35–38) that satisfactory MNC count and viability recovery can be achieved by uncontrolled-rate freezing, that is by a simple procedure of placing cells into a mechanical freezer at temperature of -90±5 C, our results pointed out that controlled-rate freezing contributed considerably to the degree of cell recovery.

Beside the cryopreservation procedure, it is well known that the choice of the cryoprotective agent and its

optimal concentration are not less important for the required cell recovery after thawing. Only two cryoprotective agents – DMSO and hydroxyethyl starch (HES) are usually used and these agents are applied in various concentrations. There are reports that low concentrations (2.2 and 4.5% DMSO with 6% HES) are sufficient for the acceptable HSPC recovery (20), and that higher DMSO concentrations (>10%), especially on higher temperatures (4° to 22° C), could be cytotoxic to human progenitors (22, 25). However, our results related to human MNC cryopreserved by Protocol 1, with 10% DMSO, revealed efficient cell recovery and viability after thawing. This finding is consistent with the reports demonstrating the lack of the cytotoxic effects of 10% DMSO on human HSPC (39), but indicating that 10% DMSO could induce decreased surface antigens expression and reduced repopulating capacity of CFU-S (40). Moreover, the data presented here demonstrated that the DMSO concentration used was not primarily related to the changes in the number or viability of HSPC, but pointed out the importance of the cryoprotector concentration in the recovery of the different progenitor cell populations. Namely, the results obtained for the murine bone marrow cells have shown that the lower (5%) DMSO concentration was efficient for the cryopreservation of the mature progenitors, CFU-GM and CFU-Sd12, while the more primitive stem cells (pre-CFU-S) could only be well cryopreserved with a higher (10%) concentration of DMSO. Thus, the adequate DMSO concentration should be applied in respect of the progenitor cell population needed to be preserved.

The efficacy of Protocol 1, used for the cryopreservation of human MNC was finally evaluated through the engraftment potential of cryopreserved cells, since these MNC were used for autologous peripheral blood stem cell transplantation. The treated patients were reinfused with 12.1 ml/kg of cell suspension immediately after thawing, without the removal of residual DMSO. Premedication was achieved by intravenous administration of corticosteroids and antihistaminics, with stimulated diuresis. No adverse effects of the infused cryoprotective agent present in thawed cells were noted and the early posttransplantation period passed without complications. The engraftment, i.e. through the reconstitution of hematopoiesis (peripheral blood leukocyte count exceeding $1x10^9$/l and platelet count $\geq 20x10^9$/l) was observed on the 11th day after transplantationfollowing the after autologous bone marrow stem cell transplantation, the hematologic recovery was achieved on day 19 (3).

Conclusion

Although there are conflicting reports regarding the cryopreservation of HSPC using the controlled-rate and noncontrolled-rate freezing-procedures, the results obtained for murine bone marrow cells clearly demonstrated much better recovery of all tested progenitor cells by the controlled-rate freezing-procedures, taking into consideration the release of the fusion heat (protocols 1 and 4). However, even this procedure has to be combined with the optimal DMSO concentration which differs depending on the maturity of the progenitor cells, since the best recovery for the mature progenitors (CFU-GM and CFU-Sd12) was achieved with Protocol 4 (5% DMSO) and for the more primitive progenitors MRA (pre CFU-S) with Protocol 1 (10% DMSO).

The presented data confirmed that the cryopreservation procedures used for the murine bone marrow cells could also be applicable for the cryopreservation of human MNC. Controlled-rate freezing procedure enabled better recovery of MNC harvested from both healthy subjects and patients. When this cryopreserved MNC were used for autologous peripheral blood stem cell transplantation, patients' hematologic recovery was achieved on day 11, confirming the significant advantages of controlled-rate freezing.

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Апстракт

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ИСПИТИВАЊЕ ОПОРАВКА КРИОКОНЗЕРВИСАНИХ МАТИЧНИХ ЋЕЛИЈА ХЕМАТОПОЕЗЕ НАМЕЊЕНИХ ТРАНСПЛАНТАЦИЈИ

Ефикасност пет протокола криоконзервације испитивана је на основу опоравка ћелија костне сржи мишева и хуманих мононуклеарних ћелија периферне крви (МНЦ) после одмрзавања. Код мишева, одређиван је опоравак веома примитивних плурипотентних хематопоезних прогенитора (МРА), плурипотентних и опредељених прогенитора (ЦФУ–ГМ и ЦФУ–Сд12). Опоравак МРА, ЦФУ–Сд12 и ЦФУ–ГМ варирао је у зависности од врсте примењене процедуре замрзавања и концентрације диметил сулфоксида (ДМСО). При извођењу процедуре програмираног замрзавања констатован је бољи опоравак свих категорија прогенитора. Програмирано замрзавање са компензацијом ослобођене топлоте фузије и употребом 5% ДМСО (протокол 4) омогућило је најбољи опоравак ЦФУ–ГМ (73,0±8,8%) и ЦФУ–Сд12 (90,0±15,9%). Супротно томе, опоравак веома примитивних МРА био је бољи (79,8±13,5%) уколико је примењена већа концентрација (10%) ДМСО у комбинацији са петостепеном процедуром замрзавања (протокол 1). Ови резултати указују на неопходност примене програмираног замрзавања при криоконзервацији појединих категорија хематопоезних прогенитора. Утврђено је да ефикасна криоконзервација МРА, али не и мање примитивних прогенитора (ЦФУ–С, ЦФУ–ГМ), захтева употребу веће концентрације ДМСО. Све то указује на могуће постојање неког механизма који изазива редукцију интрацелуларне концентрације ДМСО у ћелијама МРА, али не и у мање примитивним прогениторима. Код хуманих МНЦ, одређиван је опоравак ћелија након одмрзавања и степен реконституција хематопоезе након аутологне трансплантације. Криоконзервација по протоколу 1 је резултовала бољим опоравком хуманих МНЦ (82,7±10,4%) од протокола 3 (49,9±15,1%). Просечни опоравак МНЦ (прикупљених од болесника за аутологну трансплантацију) износио је 78,5±7,3% (протокол 1), односно 53,1±26,2% (протокол 3). Постигнути високи степен опоравка одмрзнутих ћелија и брза реконституција хематопоезе (11. дан после трансплантације) код испитиваних болесника су потврдили да програмирано замрзавање у комбинацији са оптималном концентрацијом ДМСО омогућава ефикасну заштиту прогенитора од криооштећења.

Кључне речи: **hematopoezne stem ćelije; kriokonzervacija; transplantacija hematopoeznih stem ćelija; dimetilsulfoksid; citološke tehnike; kostna srž, ćelije; protokoli, klinički.**