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UČINAK KRIOPOHRANE NA PREŽIVLJENJE KRVOTVORNIH MATIČNIH STANICA I SUBPOPULACIJA LEUKOCITA PRIKUPLJENIH IZ PERIFERNE KRVI POSTUPKOM LEUKAFEREZE

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THE EFFECT OF CRYOPRESERVATION ON RECOVERY OF HEMATOPOETIC STEM CELLS AND LEUKOCYTES SUBPOPULATION COLLECTED FROM PERIPHERAL BLOOD BY LEUKAPHERESIS

DOCTORAL DISSERTATION

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SAŽETAK

Zbog očekivanog daljnjeg razvitka i primjene pripravaka za staničnu terapiju, potrebno je poznavati čimbenike koji utječu na vijabilnost i preživljenje stanica nakon kriopohrane. Cilj ovog prospektivnog istraživanja bio je u uzorcima autolognih i alogenih pripravaka stanica prikupljenih iz periferne krvi leukaferezom odrediti broj živih krvotvornih matičnih stanica (KMS) i subpopulacija leukocita 7-amino aktinomicin D (7-AAD)/aneksin V metodom i standardnom 7-AAD metodom te ispitati utjecaj kriopohrane i različitih čimbenika na preživljenje odmrznutih stanica, a i vremensko razdoblje od odmrzavanja koje je prihvatljivo za određivanje vijabilnosti stanica u svrhu laboratorijske procjene kvalitete pripravaka stanica.

U 30 autolognih i 30 alogenih uzoraka pripravaka stanica „*single-platform*“ metodom određen je broj živih KMS-a, limfocita T i B, NK stanica i monocita. Stabilnost odmrznutih uzoraka ispitana je u tri točke: odmah po odmrzavanju te sat vremena i tri sata nakon odmrzavanja.

Vijabilnosti svih populacija stanica određene 7-AAD/aneksin V metodom bile su statistički značajno niže u odnosu na 7-AAD metodu u svježim i odmrznutim uzorcima autolognih i alogenih pripravaka, no dobivene razlike nemaju utjecaj na laboratorijsku procjenu kvalitete pripravaka KMS.

U autolognim pripravcima kriopohrana najmanje utječe na preživljenje CD3+/CD8+ stanica, a u alogenima na limfocite B (CD19+ stanice). U autolognim i alogenim pripravcima kriopohrana najviše utječe na postotak preživljenja CD34+ stanica, a kod subpopulacija leukocita na preživljenje monocita (CD14+ stanica) i u autolognim i alogenim pripravcima.

U autolognim pripravcima na preživljenje CD3+ i CD3+/CD4+ stanica negativno je utjecala viša koncentracija trombocita, a na preživljenje CD3+/CD8+ stanica duljina pohrane svježeg pripravka prije kriopohrane. U alogenim pripravcima postotak granulocita negativno je utjecao na preživljenje limfocita T.

Ispitivanjem utjecaja kriozaštitne otopine na vijabilnost stanica nakon odmrzavanja dobiveno je da se apsolutni broj živih KMS-a, limfocita B i monocita statistički značajno smanjuje već sat vremena od odmrzavanja te je pripravke s tim vrstama stanice potrebno transplantirati odmah po odmrzavanju.

U ovom istraživanju zapaženo je različito preživljenje KMS-a i subpopulacija leukocita nakon kriopohrane. Stoga bi svaki transplantacijski centar trebao u svojim uvjetima ispitati kako kriozaštitna otopina i ostali čimbenici utječu na vijabilnost i preživljenje stanica od interesa.

Ključne riječi: transplantacija, krvotvorne matične stanice, kriopohrana, stanična terapija, vijabilnost

EXTENDED SUMMARY

Background: Peripheral blood stem cell (PBSC) transplantation is a widely used therapeutic option in malignant and non-malignant hematological diseases, immunodeficiency as well as some solid tumors and autoimmune diseases. Autologous PBSCs are always cryopreserved immediately after collection, while allogeneic products are usually transplanted fresh unless the cells cannot be delivered to the recipient within 72 hours of collection. The post-thaw cell recovery is influenced by many factors, e.g. cell type, pre-freeze storage conditions, time between collection and cryopreservation, centrifugation, type and concentration of cryopreservation solution, freezing/thawing rate and storage temperature. It is therefore important to examine the effect of the aforementioned factors, especially for allogeneic hematopoietic stem cell (HSC) products, which have been cryopreserved more frequently as of recently to mitigate the donor and travel risks associated with the COVID-19 pandemic. Cryopreservation of allogeneic products was a challenge for transplant centers because until then it was uninvestigated how the viability of allogeneic products was affected by cryopreservation and thawing. Due to the expansion of cell therapy using not only HSC but also other leukocyte subpopulations in personalized medicine, the influence of these factors on the post-thawed recovery of other cell types needs to be examined to ensure the quality of cell therapy products.

Although the use of cryoprotectant dimethyl sulfoxide (DMSO) is the gold standard in cryopreservation of HSCs, it is well known that it has a negative effect on cell viability during cryopreservation as well as post-thaw. Several studies investigated how cryopreservation affects the post-thaw recovery of HSC and leukocyte subpopulations in PBSC products, while the stability of thawed PBSC samples was investigated only for CD34+ cells, and there are no data on how the length of exposure to DMSO affects viability of other leukocyte subpopulations.

According to the Joint Accreditation Committee of the International Society for Cellular Therapy and the European Group for Blood and Marrow Transplantation (JACIE) standards, viable CD34+ cell count must be analyzed in fresh PBSC products as well as in the thawed cryopreserved samples for graft quality assessment. The method with 7-aminoactinomycin D (7-AAD) is routinely used to determine viable CD34+ cells by flow cytometry and can also be used to analyze the viability of different subpopulations of leukocytes. However, the limitation of the 7-AAD method is the ability to only determine cells in necrosis and late apoptosis, but not cells in early apoptosis with an intact cell membrane, which may lead to the

overestimation of graft cell viability. However, the cells are already losing functionality in early apoptosis, and the first changes are detected on the cell membrane, resulting in the exposure of phosphatidylserine residues from the inner (cytoplasmic) leaflet to the outer leaflet of the plasma membrane. These changes in early apoptotic cells can be detected by annexin V because it has a high affinity for surface exposed phosphatidylserine.

In order to prevent further loss of viability after thawing, it is recommended to infuse the thawed cell products as soon as possible, preferably within 10 to 20 minutes. However, in some situations, the thawed cell products cannot be transplanted immediately, like in the case of the deterioration of patient's condition, or technical problems during transplantation. Also, there are situations in which the product sample taken for the control of post-thaw viability cannot be delivered immediately to the laboratory. Hence it is important to examine how the length of exposure of the cells to DMSO after thawing affects their viability, as well as to evaluate the sample post-thaw stability to determine the period in which it is possible to accurately assess the quality of the thawed cell product. It is recommended, according to good laboratory practice and International Organization for Standardization (ISO)15189:2012 (Medical laboratories—Requirements for quality and competence), to examine the stability of the samples which will be routinely analyzed to provide guidance for the management of samples for each specific analysis. Due to the expansion of the cell therapy to other leukocyte subpopulations besides CD34+ and CD3+ cells, it is also important to examine the impact of cryopreservation on all leukocyte subpopulations in autologous and allogeneic products for better assessment of the quality of cell products used as immune effector cell therapy.

Aim: The primary aim of this prospective study was to evaluate the post-thaw recovery of HSC and leukocyte subpopulations using the flow cytometry 7-AAD and 7-AAD/annexin V methods. The secondary aim was to assess how the post-thaw recovery is affected by various parameters like the cell composition and concentration in fresh leukapheresis products and the length of time from collection to cryopreservation. The third aim was to examine how the time of exposure to cryoprotective solution containing DMSO after thawing affects the sample stability and viability of HCS and leukocyte subpopulations in autologous and allogeneic PBSC samples. Additionally, the influence of the type of donor, as well as pre-cryopreservation storage time on the post-thaw viability during stability study were evaluated.

Materials and methods

Collection of autologous and allogeneic PBSCs

Thirty autologous and 30 allogeneic PBSC products cryopreserved in the Department of Transfusion Medicine and Transplantation Biology, University Hospital Centre Zagreb were included in the study. Patients (14 males, 16 females) with multiple myeloma (N=21), non-Hodgkin lymphoma (NHL) (N=8) and Hodgkin lymphoma (N=1) collected cells for autologous transplantation using disease-specific chemotherapy and granulocyte colony stimulating factor (G-CSF) filgrastim for mobilization protocols. If there was no adequate increase in the number of CD34+ cells in peripheral blood after mobilization, plerixafor was used as well. For allogeneic transplantation, PBSC products were collected from 7 related and 23 unrelated donors, of which 9 were females and 21 males. The median age of patients for autologous transplantation was 57 years (range 27 – 65 years), while for healthy donors was 32 years (range 21 -59 years).

Peripheral blood stem cells were collected using Spectra Optia apheresis system, CMNC protocol (software 11, Terumo BCT, Colorado, USA). Immediately after the collection, an aliquot of the fresh product was taken for the graft quality assessment.

Cryopreservation procedure

Apheresis products were cryopreserved using cryoprotective solution containing a mixture of 5% human albumin and dimethyl sulfoxide (DMSO) in a final concentration of 10%. The cells were frozen in a controlled programmed freezer Planer Cryo 560-16. After the addition of DMSO, aliquots were taken from each apheresis product for graft analysis. Aliquots and products in cryobags were frozen simultaneously under the same conditions and stored in the vapor phase of liquid nitrogen storage tank. Cryopreserved aliquots were stored for a minimum of 48 hours, and then thawed in a water bath at 37˚C for 1-3 minutes.

Immunophenotype analysis

Acquisition and analysis of samples were performed on flow cytometer BD FACS Canto II (BD Biosciences, San Jose, California) using BD FACS Diva software. Samples were prepared according to the manufacturer's instructions. For each sample 100 000 CD45+ cells were acquired, while samples with low CD45+ cell count were acquired up to maximum of 10 minutes. Before labelling, no sample manipulation (e.g. DMSO removal, erythrocyte lysis) was performed. The modified protocol of International Society of Hematotherapy and Graft

Engineering (ISHAGE) and single-platform method were used for CD34+ cell count determination. Leukocyte subpopulations were determined using single-platform method. Monoclonal antibodies CD3-APC, CD4-PE and CD8-FITC were used for T lymphocytes, CD19-APC for B lymphocytes, while BD Simultest (CD3-FITC, CD56/16-PE) for the determination of NK cells. Monocytes were determined using CD14-PE. The viability detection was performed using both the 7-AAD method and 7-AAD/annexin V methods. All reagents used were from Becton Dickinson (BD Biosciences, San Jose, California).

Cells were labelled in duplicate for each sample by adding an equal volume of monoclonal antibodies into BD Trucount Tube and incubating for 15 minutes at room temperature (RT) for fresh leukapheresis samples and at +4˚C for thawed samples. Because the leukapheresis sample hematocrit was low, the lyse step was not necessary, so after 15 minutes incubation period, samples were ready for acquisition on flow cytometer.

For the stability study, immediately after thawing 100 μL of sample was taken for analysis. The rest of the sample was stored at 4 ºC for further analysis. One and three hours after thawing, an aliquot of sample was taken again, and analyses were performed again. The cells were labeled using monoclonal antibodies according to flow cytometry protocols and incubated at +4 ºC for 15 minutes, without lysing due to low hematocrit in the samples. Prior to the acquisition on the flow cytometer, phosphate-buffered saline was added to each sample. At all measurement time points, cells were labeled and analyzed in duplicate for each sample.

CFU assay

Cells were plating at a concentration of $1x10^5$ nucleated cells/mL in semi-solid medium using the MethoCult H4034 (StemCell Technologies, Vancouver, BC, Canada). They were incubated under controlled conditions at a temperature of 37 °C in an atmosphere with 5% CO_2. Colonies were counted after 14 days on a StemVision analyzer (StemCell Technologies, Vancouver, BC, Canada). The results were expressed as the number of colonies per $1x10^5$ seeded nucleated cells.

Results: In autologous PBSC products, monocytes were the most, while B lymphocytes were the least abundant leukocyte subpopulation. No B lymphocytes were found in seven products (six collected from NHL patients and one from multiple myeloma patient), and there were no NK cells in one of them collected from NHL patient. Median storage time for fresh autologous and allogeneic PBSC was 4.5 (IQR 4 – 5) and 27 (IQR 6 - 48) hours, respectively.

Only six (20.0%) autologous and 11 (36.7%) allogeneic PBSC were stored overnight, while others were cryopreserved within 5 hours of collection.

In fresh autologous PBSC samples, for all cell populations the medians of viability were ≥ 99.5% using the 7-AAD method and ≥ 99.4% using the 7-AAD/annexin V method. In thawed samples medians of viability were ≥ 94.2% for the 7-AAD method and ≥92.5% for the 7-AAD/annexin V method. Statistical analysis showed a significant difference in cell viability using the 7-AAD and the 7-AAD/annexin V method for all cell populations in both autologous fresh and thawed samples. In allogeneic PBSC products the median of the viability of all cell populations in fresh samples was ≥ 99.3% with the 7-AAD method and ≥ 98.6% with the 7-AAD/annexin V method, while in thawed samples was ≥ 93.9% and ≥ 92.1% for methods with 7-AAD and 7-AAD/annexin V, respectively. A statistically significant difference between the methods for cell viability testing in allogeneic PBSC samples was obtained for all analyzed cell populations, whether it was fresh or thawed sample. In fresh and thawed autologous PBSC samples, the statistically significant difference between total cell counts with two viability methods was observed for all cell populations, while in allogeneic leukapheresis sample results showed statistically significant difference in viability for total cell counts of all populations except for CD16+56+ in fresh samples (P=0.126).

Post-thaw recovery for each individual cell population was calculated using the total number of viable cells determined using both the 7-AAD method and the 7-AAD/annexin V method. In autologous products, cryopreservation has the least effect on the post-thaw recovery of CD3+/CD8+ cells, while in allogenous products on B lymphocytes (CD19+ cells). In autologous and allogeneic products, cryopreservation has the greatest effect on the recovery of CD34+ cells, as well as on monocytes (CD14+ cells) in both autologous and allogeneic PBSC products.

For autologous PBSC samples statistical analysis showed significant differences between recoveries for CD34+ cells (P<0.001), CD19+ cells (P=0.037), CD14+ cells (P<0.001) and CD16+56+ cells (P=0.002). In allogeneic PBSC samples statistically significant differences were found between post-thaw recoveries for CD34+ and CD16+/56+ cells, P=0.002 and P=0.001, respectively. A weak correlation was obtained between the CD34+ cell recovery and recovery of CFU colonies (7-AAD method: P = 0.021, correlation coefficient 0.298; 7-AAD/annexin V method P = 0.043, coefficient correlation 0.262).

In autologous products we observed weak negative correlation between platelets and CD3+ cell recovery when viable cells were determined with the 7-AAD method (P=0.042, r2= -0.374), and platelets and CD3+CD4+ cell recovery when viable cells were determined using the 7-AAD/annexin V method (P=0.043, r2 -0.372). The correlations between storage time and recovery of CD3+CD8+ cells were weak negative using the 7-AAD method (P=0.028, r2 =-0.401) and the 7-AAD/annexin V method (P=0.028, r2 =-0.401). In allogeneic products, strong negative correlation was observed between percentage of granulocytes in fresh samples, and T cell subpopulations recoveries CD3+ cells: P<0.001, r2= -0.759 for the 7-AAD method, P<0.001, r2= -0.768 for the 7-AAD/annexin V method; CD3+CD8+ cells: P=0.001, r2= -0.668 for the 7-AAD method, P=0.001, r2= -0.666 for the 7-AAD/annexin V method; CD3+CD4+ cells: P<0.001, r2= -0.718 for the 7-AAD method, P<0.001, r2= -0.720 for the 7-AAD/annexin V method.

In stability study analysis of the viable absolute cell count showed a statistically significant difference between all measurement points for CD34+ (P<0.001), CD14+ (P<0.001) and CD19+ cells (P<0.001). For the absolute count of viable CD16+/56+ cells, the results after three hours were significantly different from the baseline results (P=0.002) as well as the results after one hour (P=0.007). In T-lymphocyte subpopulations statistically significant difference was observed between baseline results and the results obtained one-hour post-thaw (P=0.010), as well as between baseline and three hours' post-thaw (P<0.001) for absolute count of viable CD3+ cell. However, comparing the percentages of post-thaw viable cells, statistically significant difference was observed for the results between one and three hours (P=0.003). Statistically significant difference was observed for viable absolute CD3+/CD8+ cells for results obtained immediately after thawing and those after one (P<0.001) and three hours (P<0.001), as well as for the percentage of viable CD3+/CD8+ cells between the results after three hours compared to the other two measurement points (baseline vs. after three hours P=0.043, after one hour vs. after three hours P=0.003). Statistically significant difference was observed between baseline and after three hours results for absolute count of CD3+/CD4+ cells (P<0.001).

The composition of autologous and allogeneic products differed depending on the type of leukocyte subpopulations. The absolute count of viable CD14+ cells and the T-lymphocyte subpopulation were higher in autologous products compared to allogeneic products, while allogeneic products contained a higher number of viable CD34+, CD19+ and CD16+/56+ cells compared to allogeneic products. The percentage of viable CD19+ cells as well as

absolute viable CD19+ cells were significantly different between autologous and allogeneic PBSC samples in all measurement time points (baseline $P<0.001$, after one-hour $P<0.001$, after three hours $P<0.001$). Significant difference was obtained for the absolute count of viable CD14+ and CD16+/56+ cells in all measurement time points, and after one hour results for the percentage of viable CD14+ cells ($P=0.019$). Baseline results for percentage of viable CD3+, CD3+/CD8+ and CD3+/CD4+ cells showed statistically significant difference between autologous and allogeneic samples. Also, significant differences were observed for absolute viable CD3+ cells ($P=0.016$) and CD3+/CD8+ cells ($P=0.031$) one-hour post-thaw.

Post-thaw stability of allogeneic cryopreserved PBSC samples were analyzed between leukapheresis products stored up to 24 hours (N=20), and those stored more than 24 hours before cryopreservation (N=10). No statistically significant differences were observed for none of the viable absolute cell counts in all leukocyte subpopulations, except for viable CD3+/CD4+ cells determined after three hours' post-thaw ($P=0.028$). Statistically significant differences were observed in percentage of viable CD14+, CD19+ and CD16+/56 cells for baseline results, as well as in percentage of viable CD16+/56+ cells one hour after thawing. In analysis of T-lymphocyte subpopulations, statistically significant difference was obtained for the percentage of viable cells between all measurement time points.

Conclusions: Although the differences between results obtained using the 7-AAD/annexin V method compared to the 7-AAD method were statistically significant, they did notinfluence the laboratory graft quality assessment, and the use of the 7-AAD method was shown to be suitable enough for determining the cell viability and assessing post-thaw cell recovery in the routine quality control of PBSC samples. Due to the increasing need for the use of various types of cell therapy, it is necessary to further identify the factors affecting the cell viability and post-thaw recovery, such as the characteristic of the apheresis products and pre-freeze storage conditions which can adversely affect the quality of the graft. The results showed that cryopreservation had different effects on viability and stability of various leukocyte subpopulations in thawed PBSC samples. The type of donors, as well as the length of storage before cryopreservation did not affect cryopreserved PBSC sample post-thawing stability. In PBSC samples the viability of HSC, T and B lymphocytes, monocytes and NK cells significantly decreased immediately post-thawing, therefore cellular products containing them must be infused immediately after thawing in order to prevent the further loss of cell viability and preserve the quality of the graft. Since different viabilities and post-thaw recoveries of leukocyte subpopulations were observed in our study, the transplantation centers should

evaluate how cryopreservation and other factors affect the viability and recovery of each cell population of interest in their settings.

Key words: cell therapy, cell viability, peripheral blood stem cells, cryopreservation