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Original research paper

The impact of cryoprotectant exposure time on post-thaw viability of autologous and allogeneic hematopoietic stem cells and leukocyte subpopulations

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ABSTRACT

Although the use of cryoprotectant dimethyl sulfoxide (DMSO) is the gold standard in cryopreservation of hematopoietic stem cells, it is well known that it has a negative effect on cell viability. The aim of this prospective study was to examine how the length of post-thaw exposure to DMSO affects the cell viability and stability of peripheral blood stem cell (PBSC) samples. Additionally, the effects of donor type and pre-cryopreservation storage time on post-thaw viability during the stability study were evaluated. In 30 autologous and 30 allogeneic PBSC samples viable CD34+, CD14+, CD19+, CD16+/56+, and CD3+ cells were determined immediately after thawing, and one- and three-hours post-thaw.

Analysis of the absolute count of viable cells in thawed samples showed a significant difference between all measurement points for CD34+ ($p < 0.001$), CD14+ ($p < 0.001$), and CD19+ cells ($p < 0.001$). No significant differences were observed for post-thaw stability of allogeneic samples analysed between products stored before cryopreservation ≥ 24 hours (N = 20), and those stored < 24 hours (N = 10), except for viable CD3+/CD4+ cells after three hours post-thaw ($p = 0.028$). In conclusion, DMSO had different effects on leukocyte subpopulations in cryopreserved PBSC samples. The type of donors and the length of storage before cryopreservation did not affect the post-thaw stability of cryopreserved PBSC samples.

Keywords: cryopreservation, sample stability, viability, peripheral blood stem cells

The essential part of the assessment of the quality of hematopoietic stem cell (HSC) products is the determination of viable CD34+ cells. According to the Joint Accreditation Committee of the International Society for Cellular Therapy and the European Group for Blood and Marrow Transplantation (JACIE) standards, CD34+ cell content and viability

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must be analyzed in fresh peripheral blood stem cell (PBSC) products as well as in the thawed cryopreserved samples for graft quality assessment (1).

The analysis of the viability of cryopreserved autologous PBSC products is a routine part of quality control before the transplantation, while allogeneic products were rarely cryopreserved because they were mostly transplanted fresh (2). At the time of the Coronavirus Disease 2019 (COVID-19) pandemic, cryopreservation of allogeneic products was also required. This was a challenge for transplant centers because until then it was uninvestigated how the viability of allogeneic products was affected by cryopreservation and thawing (3, 4).

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 after thawing (5). Several studies investigated how cryopreservation affects the post-thaw recovery of HSC and leukocyte subpopulations in PBSC products (6, 7). The stability of thawed PBSC samples was investigated only for CD34+ cells, while there is no data on how the length of exposure to DMSO affects the viability of other leukocyte subpopulations (8). 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 the purpose of better assessment of the quality of cell products used as immune effector cell therapy.

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 (2). However, in some situations, the thawed cell products cannot be transplanted immediately, like in the case of the deterioration of the patient's condition, or the 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 (9, 10).

The aim of this prospective study 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 the stability study was evaluated.

EXPERIMENTAL

Materials

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 (male 14, female 16) with multiple myeloma (N = 21), non-Hodgkin lymphoma (N = 8), and Hodgkin lymphoma (N = 1) collected cells for autologous transplantation. For allogeneic transplantation, PBSC products were collected from 7 related and 23 unrelated donors, of which 9 were female and 21 male. 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).

Cryopreservation and thawing of PBSC samples

All autologous and 10 allogeneic PBSC products collected in our institution were cryopreserved within 24 hours, while 20 allogeneic products received from World Marrow Donor Association (WMDA) collection centres were stored before the cryopreservation > 24 hours.

PBSC products were cryopreserved using a cryoprotective solution containing a mixture of 5 % human albumin (Albunorm, Human albumin solution, Octapharma Handelsgesellschaft mbH, Austria) and 20 % of DMSO (CryoSure DMSO, WAK-Chemie Medical GmbH, Germany). Under controlled conditions (4 °C) the cryoprotective solution was added to the centrifuged plasma-depleted cells automatically at a flow rate of 5 mL min^{-1} using a Smart-Max device (Biosafe SA, Switzerland). The final DMSO concentration in the bag was 10 %, and the white blood cell (WBC) content was no more than 200×10^9 L^{-1}. After the addition of cryoprotectant solution and before the freezing process, an aliquot from each leukapheresis product was taken in cryotubes for graft analysis. Samples in cryotubes and products in cryobags were frozen simultaneously under the same conditions to –160 °C using the controlled rate freezer Planer Cryo 560-16 (Planer Concentrate Ltd., UK), and afterwards stored in a vapor nitrogen storage container (Consarctic, Consarctic GmbH, Germany). Before thawing and analysis, cryopreserved samples were stored for at least 48 hours.

Samples were thawed in a 37 °C water bath for 1–3 minutes with gentle agitation. The cryotubes were removed from the water bath when only a few ice crystals remained, and 100 μL of samples were taken for analysis. The rest of the sample was stored at 4 °C for further analysis. One and three hours after thawing, 100 μL of samples were taken again and analyses were performed again. The cells were labeled using monoclonal antibodies according to flow cytometry protocols and incubated at 4 °C for 15 minutes, without lysing due to low hematocrit in the samples. Prior to the acquisition on the flow cytometer, 1 mL of phosphate-buffered saline (PBS) was added to each sample. At all measurement time points, cells were labeled and analyzed in duplicate for each sample.

Flow cytometry analysis

Acquisition and analysis of samples were performed on flow cytometer BD FACS Canto II (BD Biosciences, USA) using BD FACS Diva software, version 8.0.1. (BD Biosciences). Each day before the routine, cytometer setup was performed using BD FACS Diva CS&T IVD beads (BD Biosciences).

Samples were prepared according to the manufacturer's instructions, using single-platform methods for all leukocyte subpopulations included in the study (HSC, T and B lymphocytes, NK cells, and monocytes). For each sample, 100,000 CD45+ cells were acquired.

Before labelling, no manipulation with cells (*e.g.*, DMSO removal, sample dilution) in a cryotube was performed.

For the determination of CD34+ cells, a modified single-platform International Society of Hematotherapy and Graft Engineering (ISHAGE) protocol was used (11). Leukocyte subpopulations were labeled by adding an equal volume of monoclonal antibodies into BD Troucount Tubes: CD45-FITC or BV510, CD3-FITC or APC, CD8-FITC, CD4-PE, CD19-APC, CD16/56-PE and CD14-PE. Different fluorochromes were used in different protocols: CD45-FITC was used with CD14-PE for monocyte detection, while CD45-BV510 was used in combination with CD-3 APC, CD8-FITC and CD4-FE for T-lymphocyte subpopulations detection. Gating strategies for the leukocyte subpopulation were designed following the same logic as that behind the ISHAGE recommendations for stem cell enumeration (12).

7-Aminoactinomycin D (7-AAD) was used for the assessment of cell viability. All reagents used in this study were from Becton Dickinson (BD Biosciences) and were stored at 4 °C until used.

Statistical analysis

Data distribution normality was assessed using the Shapiro-Wilk test. Results were presented as medians and interquartile ranges. The differences between the three measurement time points: immediately after thawing (baseline), one and three hours after thawing were assessed using repeated measures analysis of variance (ANOVA) for normally distributed data, and the Friedman test for non-normally distributed data.

The difference in the cell viability and absolute viable cell count between measurement time points was analyzed using the Wilcoxon test for non-normally distributed data, and the *t*-test for paired samples for data distributed normally.

The influence of the type of donors (autologous *vs.* allogeneic), as well the pre-cryopreservation storage time on the post-thaw viability of allogeneic PBSC samples), was analyzed using the Mann-Whitney test for non-normally distributed data, while for data distributed normally, the *t*-test was used. Results were considered statistically significant if $p < 0.05$. All statistical analyses were performed using Medcalc statistical software, version 14.12.0 (MedCalc, Belgium).

Ethics

The approvals for this study were obtained from the Ethics Committee of University Hospital Center Zagreb (8.1.-21/116-2; 02/21 JG) and the Ethics Committee for Experimentation of the University of Zagreb Faculty of Pharmacy and Biochemistry (251-62-03-21-27), and all patients gave their written informed consent to participate in this study.

RESULTS AND DISCUSSION

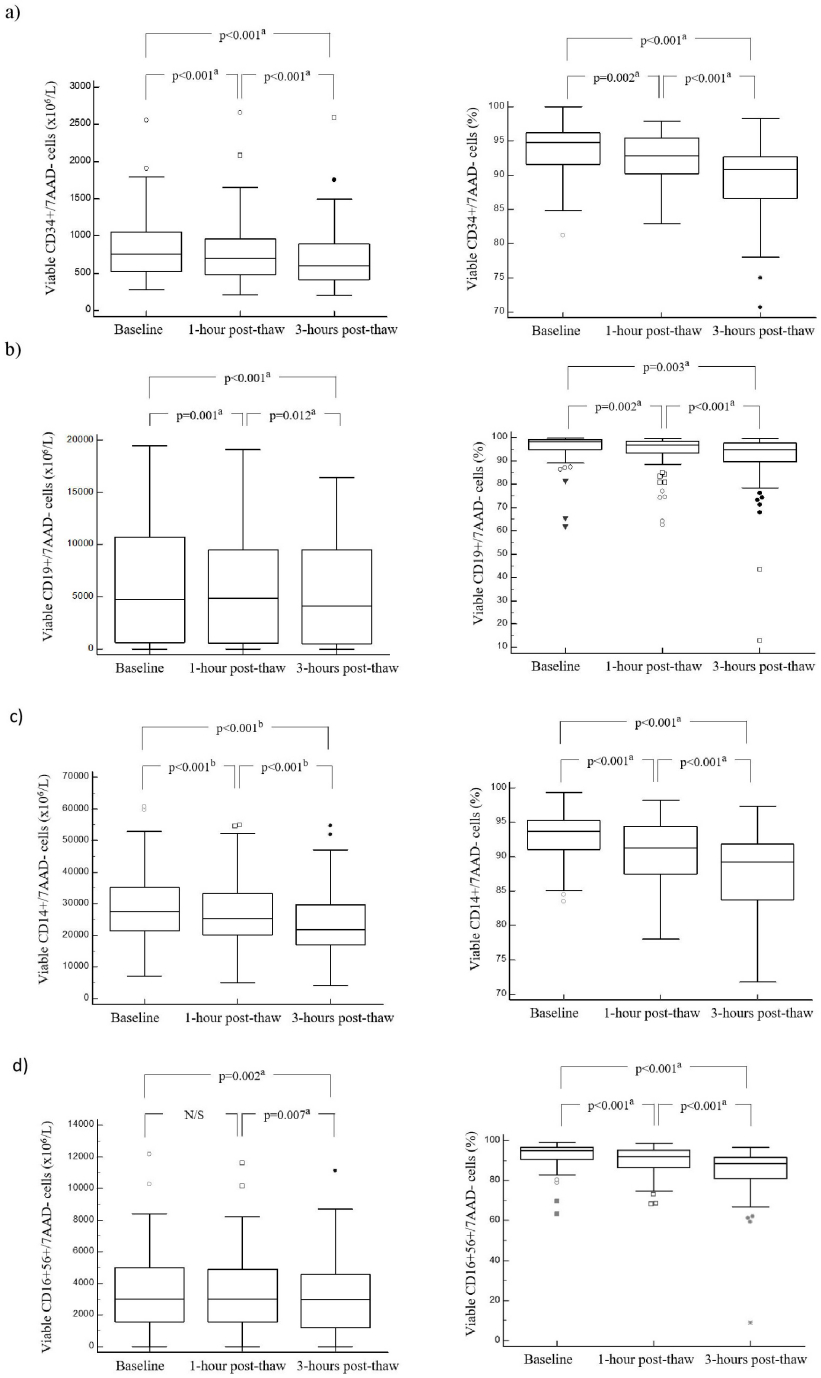
It is recommended that cryopreserved PBSCs should be infused immediately after thawing in order to minimize the detrimental effect of the cryoprotective solution on HSC viability (2). Since there are situations in which the infusion must be delayed, or problems with the postponed delivery of the post-thaw PBSC sample to the laboratory for analysis,

it is necessary to examine the influence of the length of exposure to cryoprotectant on the cell viability of thawed PBSC products. The stability of the samples should also be evaluated in order to determine the length of time after thawing at which there is no clinically significant decrease in cell viability and the samples are acceptable for analysis.

Only a few studies evaluated short-term post-thawed stability by determining the viability of CD34+ cells, mostly in UCB samples, but their results differ (13–15).

The results of our study showed statistically significant decreases in the absolute viable cell counts between all measurement points for HSCs (CD34+ cells, $p < 0.001$), B lymphocytes (CD19+ cells, $p < 0.001$), and monocytes (CD14+ cells, $p < 0.001$) (Fig. 1). The most resistant to the effect of DMSO were T lymphocytes (CD3+ cell). A 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 an absolute count of viable CD3+ cells (Fig. 1). However, comparing the percentages of viable cells, a statistically significant difference was observed only for the results between one- and three-hours post-thaw ($p = 0.003$). The analysis performed on T lymphocyte subpopulations showed that DMSO had less effect on the viability of CD3+/CD4+ cells than CD3+/CD8+ cells. The absolute number of viable CD3+/CD4+ cells significantly decreased only three hours post-thawing ($p < 0.001$), whereas viable CD3+/CD8+ cells already decreased an hour post-thawing (Fig. 1). For NK cells (CD16+/56+ cells), the statistically significant decrease of absolute count of viable cells was observed only after three hours post-thaw ($p = 0.002$) (Fig. 1). The separate analysis of autologous and allogeneic samples showed statistically significant decreases of HSCs ($p < 0.001$), monocytes ($p < 0.001$) and CD3+/CD8+ cells ($p = 0.001$) in autologous samples (Fig. 2), and HSCs ($p < 0.001$), monocytes ($p < 0.001$) and B lymphocytes in allogeneic samples ($p < 0.001$) (Fig. 3). Cell products containing aforementioned leukocyte subpopulations should be infused immediately after thawing to maintain cell viability. In autologous samples, the most resistant to the effect of DMSO were B lymphocytes whose viability statistically significantly decreased only three hours post-thaw (Fig. 2). In allogeneic samples the statistically significant decrease was observed in the percentages of viable NK cells in all three measurement points ($p < 0.001$), and in absolute count of viable CD3+ cells between baseline results and those measured three hours post-thawing (Fig. 3). Cell products containing aforementioned leukocyte subpopulations should be infused immediately after thawing to maintain cell viability. In autologous samples, the most resistant to the effect of DMSO were B lymphocytes whose viability statistically significantly decreased only three hours post-thaw (Fig. 2). In allogeneic samples the statistically significant decrease was observed in the percentages of viable NK cells in all three measurement points ($p < 0.001$), and in absolute count of viable CD3+ cells between baseline results and those measured three hours post-thawing (Fig. 3).

Lee HY *et al.* reported that the viability of CD34+cells significantly decreased 24 hours after thawing when UCB samples were stored at room temperature (RT) (13). Conversely, Fry *et al.* stated that the most suitable post-thaw procedure to obtain the highest levels of viable cells depends upon the length of time before infusion, and that the acceptable time for UCB samples is one hour after thawing if samples do not require any manipulation (*e.g.* removal of DMSO, or sample dilution) (14). Huang *et al.* concluded in their study that the post-thaw viability of UCB HPCs was preserved for less than 30 minutes at RT, and less than 20 minutes might be the optimal length of time for the infusion of cryopreserved UCB


a)
p<0.001[a]
p<0.001[a]
p<0.001[a]
Viable CD34+/7AAD- cells (x10⁶/L)
Baseline
1-hour post-thaw
3-hours post-thaw
p<0.001[a]
p=0.002[a]
p<0.001[a]
Viable CD34+/7AAD- cells (%)
b)
p<0.001[a]
p=0.001[a]
p=0.012[a]
Viable CD19+/7AAD- cells (x10⁶/L)
p=0.003[a]
p=0.002[a]
p<0.001[a]
Viable CD19+/7AAD- cells (%)
c)
p<0.001[b]
p<0.001[b]
p<0.001[b]
Viable CD14+/7AAD- cells (x10⁶/L)
p<0.001[a]
p<0.001[a]
p<0.001[a]
Viable CD14+/7AAD- cells (%)
d)
p=0.002[a]
N/S
p=0.007[a]
Viable CD16+56+/7AAD- cells (x10⁶/L)
p<0.001[a]
p<0.001[a]
p<0.001[a]
Viable CD16+56+/7AAD- cells (%)

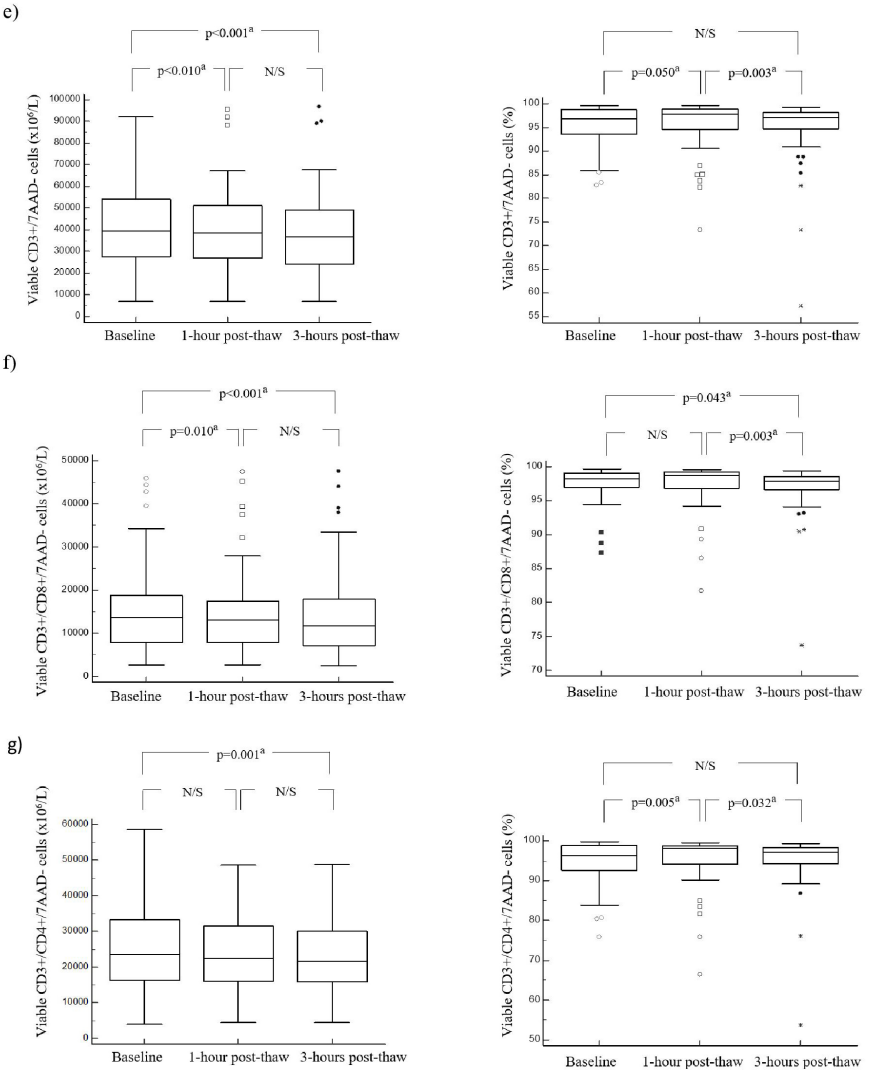
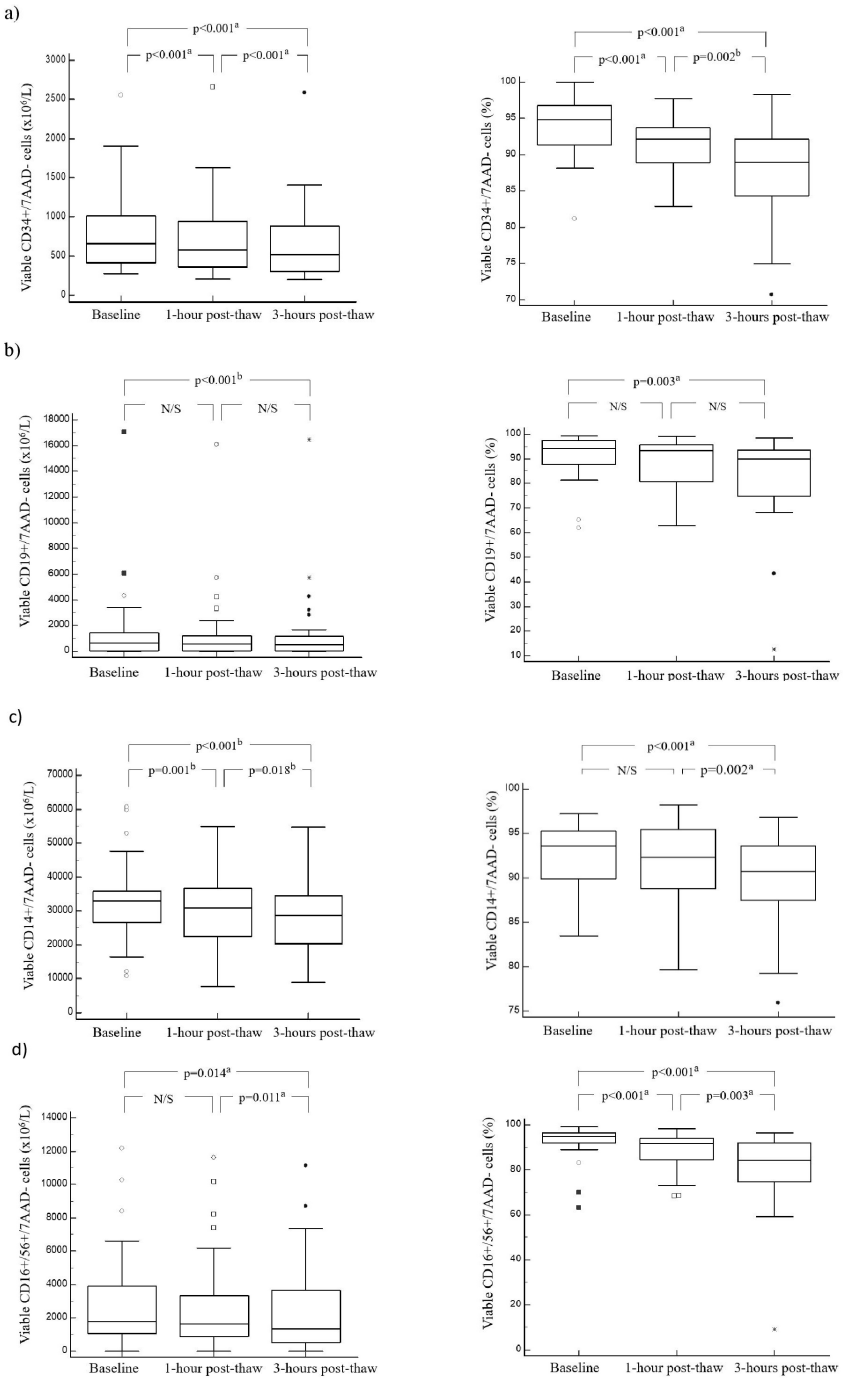


Fig. 1. Results of the stability study analysis for leukocyte subpopulations between all measurement points. a) viable CD34+ cells (absolute count $p < 0.001$ ANOVA Friedman test; percentage $p < 0.001$ ANOVA Friedman test); b) viable CD19+ cells (absolute count $p < 0.001$ ANOVA Friedman test; percentage $p < 0.001$; ANOVA Friedman test); c) viable CD14+ cells (absolute count $p < 0.001$ ANOVA repeated measures analysis of variance; percentage $p < 0.001$; ANOVA Friedman test); d) viable CD16+/56+ cells (absolute count $p = 0.005$ ANOVA Friedman test; percentage $p < 0.001$; ANOVA Friedman test); e) viable CD3+ cells (absolute count $p = 0.012$ ANOVA Friedman test; percentage $p = 0.016$ ANOVA Friedman test); f) viable CD3+/CD8+ cells (absolute count $p < 0.001$ ANOVA Friedman test; percentage $p < 0.001$ ANOVA Friedman test); g) viable CD3+/CD4+ cells (absolute count $p < 0.001$ ANOVA repeated measures analysis of variance; percentage $p = 0.057$ ANOVA Friedman test). [a] Wilcoxon test; [b] *t*-test for paired samples.


a)
p<0.001a
p<0.001a
p<0.001a
Viable CD34+/7AAD- cells (x10⁶/L)
Baseline
1-hour post-thaw
3-hours post-thaw
p<0.001a
p<0.001a
p=0.002b
Viable CD34+/7AAD- cells (%)
b)
p<0.001b
N/S
N/S
Viable CD19+/7AAD- cells (x10⁶/L)
p=0.003a
N/S
N/S
Viable CD19+/7AAD- cells (%)
c)
p<0.001b
p=0.001b
p=0.018b
Viable CD14+/7AAD- cells (x10⁶/L)
p<0.001a
N/S
p=0.002a
Viable CD14+/7AAD- cells (%)
d)
p=0.014a
N/S
p=0.011a
Viable CD16+/56+/7AAD- cells (x10⁶/L)
p<0.001a
p<0.001a
p=0.003a
Viable CD16+/56+/7AAD- cells (%)

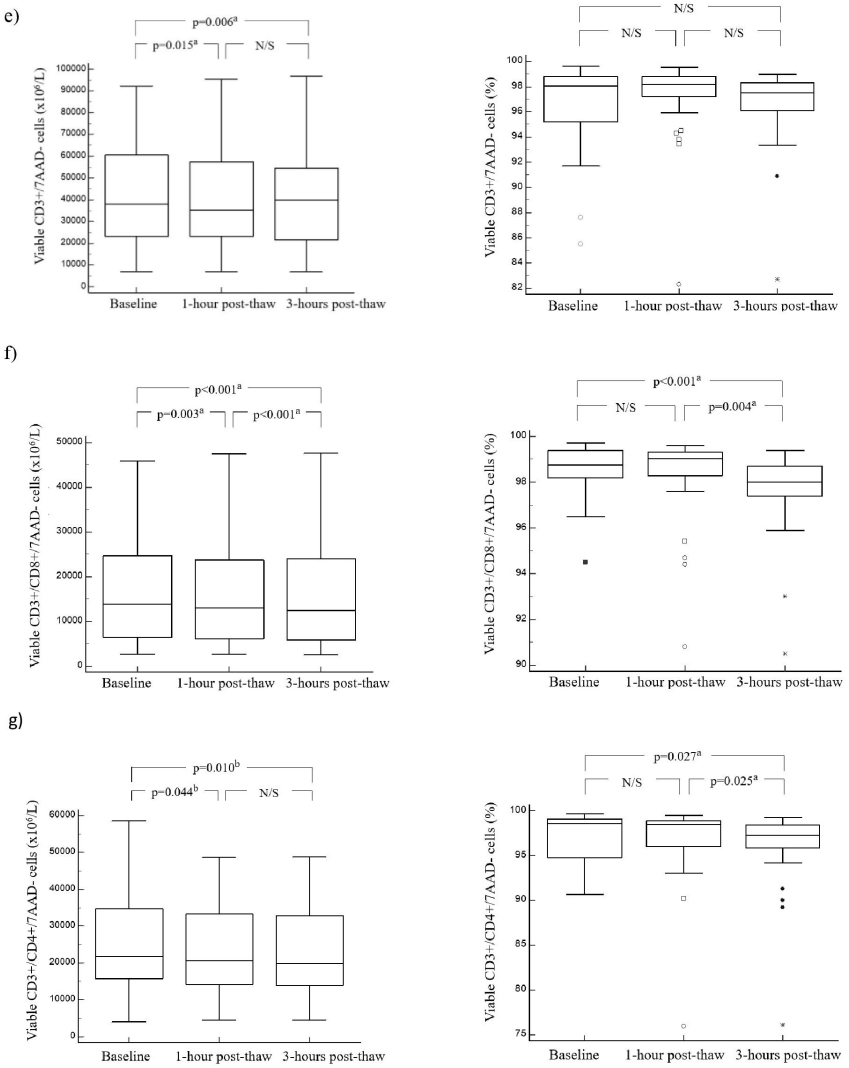
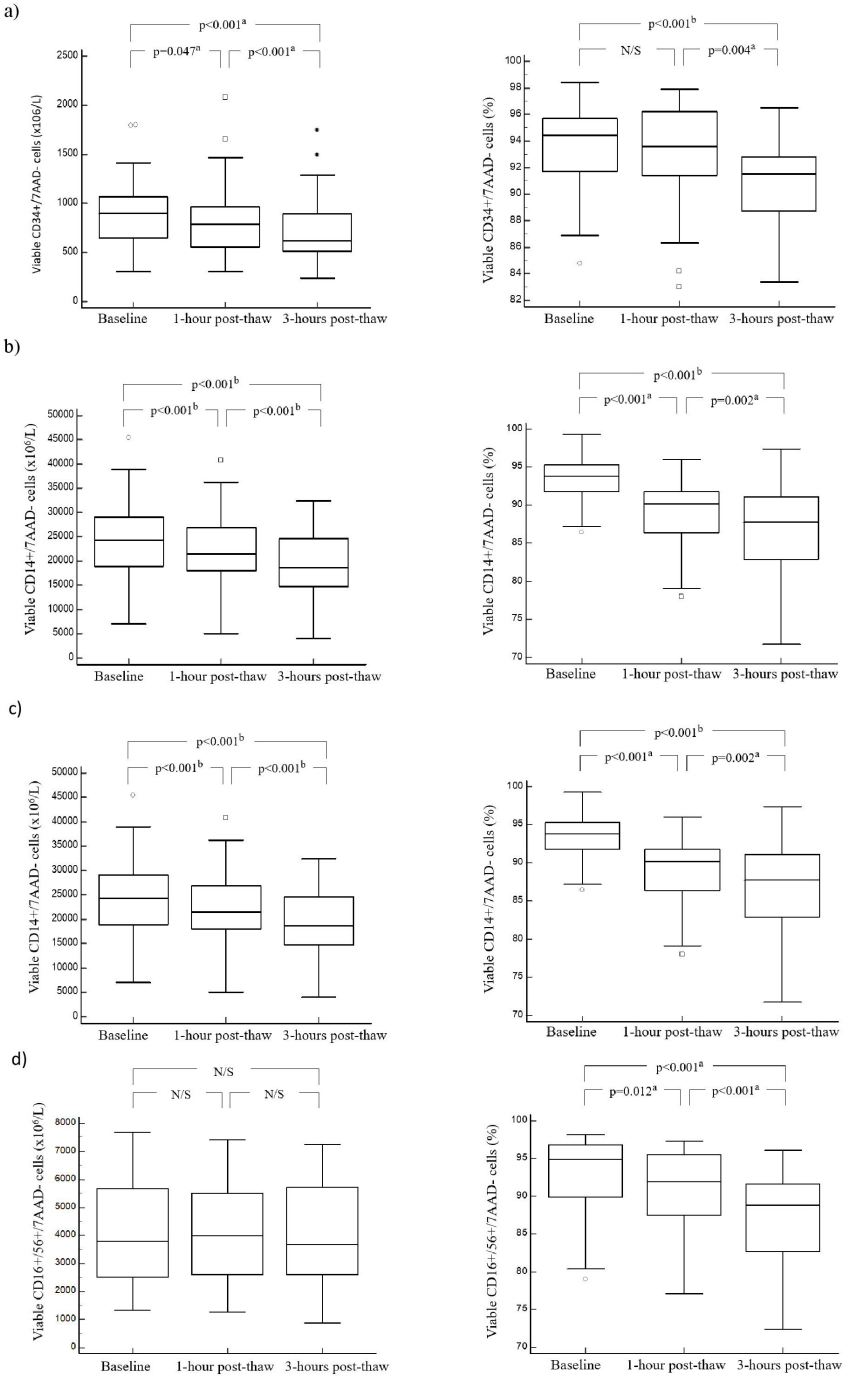


Fig. 2. Results of the stability study analysis for leukocyte subpopulations in autologous samples between all measurement points. a) viable CD34+ cells (absolute count $p < 0.001$ ANOVA Friedman test; percentage $p < 0.001$ ANOVA Friedman test); b) viable CD19+ cells (absolute count $p < 0.001$ ANOVA Friedman test; percentage $p = 0.002$; ANOVA Friedman test); c) viable CD14+ cells (absolute count $p < 0.001$ ANOVA repeated measures analysis of variance; percentage $p < 0.001$; ANOVA Friedman test); d) viable CD16+/56+ cells (absolute count $p = 0.025$ ANOVA Friedman test; percentage $p < 0.001$; ANOVA Friedman test); e) viable CD3+ cells (absolute count $p = 0.030$ ANOVA Friedman test; percentage $p = 0.051$ ANOVA Friedman test); f) viable CD3+/CD8+ cells (absolute count $p = 0.025$ ANOVA Friedman test; percentage $p < 0.001$ ANOVA Friedman test); g) viable CD3+/CD4+ cells (absolute count $p = 0.011$ ANOVA repeated measures analysis of variance; percentage $p = 0.011$ ANOVA Friedman test). [a] Wilcoxon test; [b] *t*-test for paired samples.


a)
p<0.001[a]
p=0.047[a]
p<0.001[a]
Viable CD34+/7AAD- cells (x106/L)
Baseline
1-hour post-thaw
3-hours post-thaw
p<0.001[b]
N/S
p=0.004[a]
Viable CD34+/7AAD- cells (%)
b)
p<0.001[b]
p<0.001[b]
p<0.001[b]
Viable CD14+/7AAD- cells (x10⁶/L)
p<0.001[b]
p<0.001[a]
p=0.002[a]
Viable CD14+/7AAD- cells (%)
c)
p<0.001[b]
p<0.001[b]
p<0.001[b]
Viable CD14+/7AAD- cells (x10⁶/L)
p<0.001[b]
p<0.001[a]
p=0.002[a]
Viable CD14+/7AAD- cells (%)
d)
N/S
N/S
N/S
Viable CD16+/56+/7AAD- cells (x10⁶/L)
p<0.001[a]
p=0.012[a]
p<0.001[a]
Viable CD16+/56+/7AAD- cells (%)

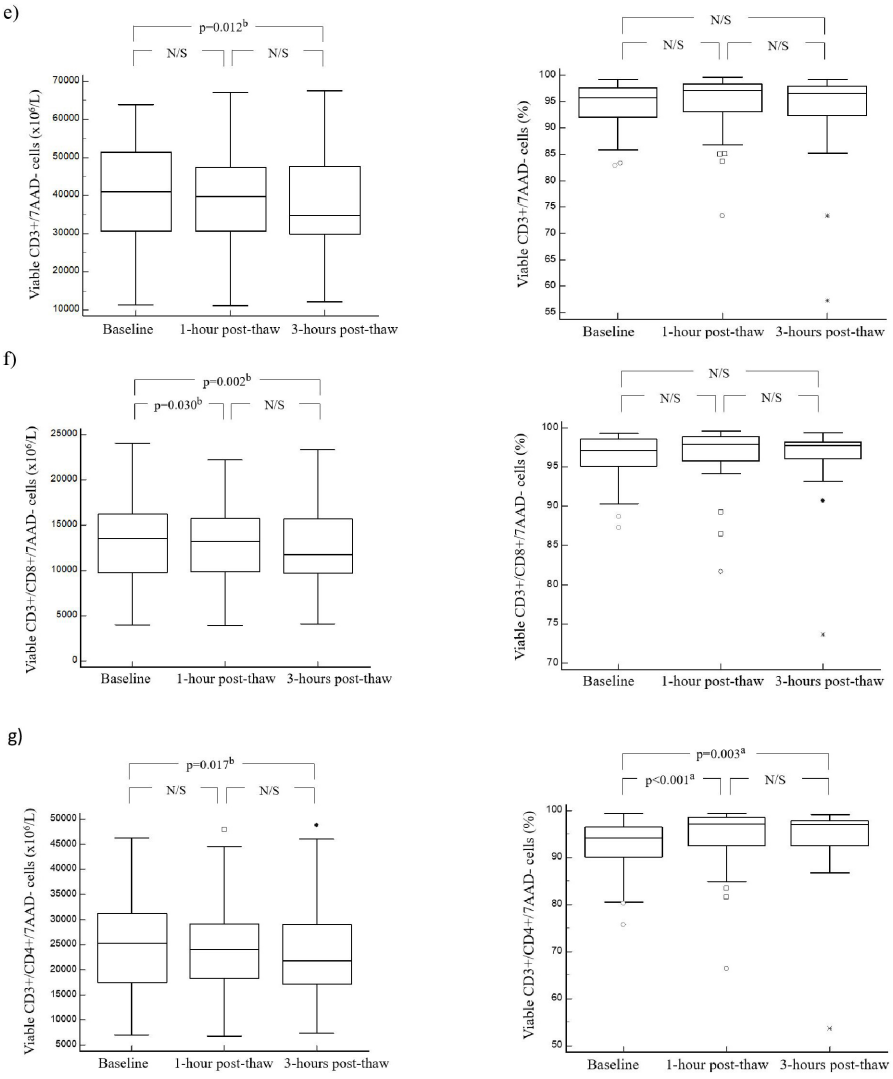


Fig. 3. Results of the stability study analysis for leukocyte subpopulations in allogeneic samples between all measurement points. a) viable CD34+ cells (absolute count $p < 0.001$ ANOVA Friedman test; percentage $p < 0.001$ ANOVA Friedman test); b) viable CD19+ cells (absolute count $p < 0.001$ ANOVA repeated measures analysis of variance; percentage $p < 0.001$; ANOVA Friedman test); c) viable CD14+ cells (absolute count $p < 0.001$ ANOVA repeated measures analysis of variance; percentage $p < 0.001$; ANOVA Friedman test); d) viable CD16+/56+ cells (absolute count $p = 0.075$ ANOVA repeated measures analysis of variance; percentage $p < 0.001$ ANOVA Friedman test); e) viable CD3+ cells (absolute count $p = 0.023$ ANOVA Friedman test; percentage $p = 0.092$ ANOVA Friedman test); f) viable CD3+/CD8+ cells (absolute count $p = 0.002$ ANOVA repeated measures analysis of variance; percentage $p = 0.125$ ANOVA Friedman test); g) viable CD3+/CD4+ cells (absolute count $p = 0.024$ ANOVA repeated measures analysis of variance; percentage $p < 0.001$ ANOVA Friedman test). [a] Wilcoxon test; [b] *t*-test for paired samples.

after thawing (15). Yang *et al.* in their study examined the effect of incubation time (every 20 minutes for 2 hours) and temperature (0, 22, and 37 °C) on the recovery and survival of CD34+ cells in autologous PBSCs and concluded that the percent of recovery and survival of CD34+ cells were consistent and unaffected by evaluated incubation time and temperatures (8). To our knowledge, there have been no studies that evaluated the short-term post-thaw stability of autologous and allogeneic PBSC samples in which the effect of the cryoprotectant on the viability of all leukocyte subpopulations has been examined.

The impact of DMSO on cell viability depends on the cell type, cell developmental stage, and cell differentiation phase, but also on the DMSO concentration and length of cell's exposure to DMSO, and in some cases, the post-thaw cryoprotectant removal was performed (16). In this study, the cryoprotective solution was not removed before infusion of thawed PBSC products nor from the samples in order to avoid cell loss during manipulation. It is important that each laboratory evaluates its own conditions of cryopreservation and thawing, for which time period there is no clinically significant change in post-thaw viability of each cell population of interest.

The difference in post-thaw cell viability between autologous and allogeneic PBSC samples is presented in Table I. The composition of autologous and allogeneic products differed depending on the type of leukocyte subpopulations. As shown in Table I, the absolute number of viable B lymphocytes and NK cells was lower in autologous samples than in allogeneic samples. In our study, no B lymphocytes were found in seven products, six were collected from NHL patients and one from multiple myeloma patients. Also, there were no NK cells in one product collected from NHL patients. A plausible explanation is that NHL patients, in addition to chemotherapy, had received rituximab, which killed normal B cells (17, 18). Therefore, it can be assumed that the difference in the leukocyte subpopulations between autologous and allogeneic products is caused by the effect of previous therapy on the presence of leukocyte subpopulation in peripheral blood, which consequently affects cell composition in autologous products.

The baseline absolute count of viable CD14+ cells and CD3+/CD8+ cells 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 autologous products. On the other hand, the analysis of the baseline percentage of viable cells, showed a statistically significant difference between autologous and allogeneic samples for CD3+, CD3+/CD8+, and CD3+/CD4+ cells, without difference in absolute count of viable cells.

The analysis of post-thaw cell viability between autologous and allogeneic PBSC samples in three measurement points showed significant differences for absolute viable CD3+ cells ($p = 0.016$) and CD3+/CD8+ cells ($p = 0.031$) one-hour post-thaw. The absolute numbers of viable monocytes (CD14+ cells) and NK cells (CD16+/56+ cells) were statistically different between autologous and allogeneic samples at all measurement points but without statistically significant difference in the percentage of viable cells of interest, except for the percentage of viable CD14+ cells one-hour post-thaw ($p = 0.019$).

Nowadays, the flow cytometry single-platform method for determining HSC and other cells in leukapheresis products has been accepted as the gold standard in routine work, in order to minimize errors in cell measurement and analysis (19). Also, the single-platform method used for the analysis of the absolute count of viable cells is more reliable because it takes into account the absolute number of viable leukocytes (CD45+ cells) while the

Table I. The difference in post-thaw cell viability between autologous and allogeneic PBSC samples in three measurement points

Cell population		Baseline (median/IQR)			After one hour (median/IQR)			After three hours (median/IQR)		
Cell population	Unit	Autologous (N = 30)	Allogeneic (N = 30)	*p*	Autologous (N = 30)	Allogeneic (N = 30)	*p*	Autologous (N = 30)	Allogeneic (N = 30)	*p*
Viable CD34	$\times 10^6$/L	655 (414–1010)	902 (647–1064)	0.214*	581 (364–947)	789 (554–970)	0.141*	523 (300 -886)	622 (511–894)	0.139*
	%	94.9 (91.4–96.8)	94.5 (91.7–95.7)	0.433*	92.2 (88.9–93.8)	93.6 (91.4–96.2)	0.080*	89.0 (84.3–92.2)	91.5 (88.7–92.8)	0.044*
Viable CD14	$\times 10^6$/L	33039 (26507–35916)	24231 (18903–29026)	**0.004****	30750 (22329–36557)	21432 (18052–26929)	**0.003****	28688 (20295–34473)	18646 (14810–24614)	**< 0.001****
	%	93.6 (89.9–95.3)	93.9 (91.8–95.3)	0.530*	92.4 (88.8–95.5)	90.2 (86.4–91.8)	**0.019***	90.8 (87.5–93.6)	87.8 (82.9–91.1)	0.033*
Viable CD19	$\times 10^6$/L	636 (34–1438)	10444 (8425–12956)	**< 0.001***	576 (32–1191)	9335 (8366–12648)	**< 0.001***	545 (29–1150)	9301 (7715–12230)	**< 0.001***
	%	94.2 (87.8–97.5)	99.1 (98.2–99.3)	**< 0.001***	93.2 (80.7–95.7)	98.1 (96.9–99.0)	**< 0.001***	89.9 (74.8–93.6)	97.2 (95.0–98.2)	**< 0.001***
Viable CD16/56	$\times 10^6$/L	1796 (1062–3909)	3796 (2513–5683)	**0.002***	1646 (844–3335)	3988 (2613–5502)	**0.003***	1362 (539–3676)	3671 (2610–5713)	**0.003***
	%	94.9 (92.1–96.6)	94.9 (89.9–96.8)	0.060*	91.8 (84.6–94.2)	91.9 (87.5–95.5)	0.396*	84.3 (74.7–92.0)	88.8 (82.7–91.7)	0.297*
Viable CD3	$\times 10^6$/L	38258 (23319–60413)	40996 (30666–51361)	0.836*	35255 (23128–57315)	39786 (30676–47518)	0.941*	40118 (21728–54671)	34766 (29906–47755)	0.971*
	%	98.1 (95.2–98.8)	95.7 (92.1–97.6)	**0.011***	98.2 (97.2–98.8)	97.2 (93.1–98.3)	**0.016***	97.5 (96.1–98.3)	96.5 (92.3–98.0)	0.069*
Viable CD3/CD8	$\times 10^6$/L	13919 (6400–24697)	13558 (9726–16183)	0.790*	13102 (6124–23765)	13168 (9851–15701)	0.976*	12430 (5930–24066)	11706 (9657–15657)	0.918*
	%	98.8 (98.2–99.4)	97.1 (95.1–98.6)	**< 0.001***	99.1 (98.3–99.3)	97.9 (95.8–98.9)	**0.031***	98.0 (97.4–98.7)	97.8 (96.1–98.2)	0.080*

Viable CD3/CD4	×10⁶/L	21765 (15633–34787)	25323 (17527–31264)	0.714**	20720 (14164–33216)	24080 (18281–29061)	0.530**	19765 (13880–32880)	21801 (17096–29041)	0.584**
	%	98.6 (94.8–99.1)	94.3 (90.1–96.5)	**< 0.001***	98.5 (96.0–98.9)	97.3 (92.6–98.5)	0.068*	97.3 (95.9–98.4)	97.1 (92.6–97.9)	0.217*

Statistically significant differences ($p < 0.05$) are marked in bold; * Mann-Whitney test; ** *t*-test.

Table II. The influence of pre-cryopreservation storage time on the post-thaw viability of allogeneic PBSC samples

Cell population		Baseline (median/IQR)			After one hour (median/IQR)			After three hours (median/IQR)		
Cell population	Unit	Storage time < 24 h (N = 10)	Storage time ≥ 24 h (N = 20)	*p*	Storage time < 24 h (N = 10)	Storage time ≥ 24 h (N = 20)	*p*	Storage time < 24 h (N = 10)	Storage time ≥24 h (N = 20)	*p*
Viable CD34	×10⁶/L	784 (620–1028)	913 (663–1071)	0.338**	819 (502–1019)	789 (602–956)	0.860*	616 (460–894)	656 (524–894)	0.482*
	%	95.0 (91.4–95.7)	93.8 (91.8–95.6)	0.775*	94.8 (92.1–96.5)	93.5 (91.1–95.7)	0.355*	91.4 (87.3–92.4)	91.6 (88.8–94.0)	0.628*
Viable CD14	×10⁶/L	20577 (14962–26572)	25049 (21855–30287)	0.153**	19825 (14952–21770)	23951 (18756–27341)	0.177**	15848 (11244–18363)	20011 (16752–25124)	0.107**
	%	95.6 (94.7–97.1)	92.9 (90.6–94.3)	**0.004***	91.7 (90.0–94.6)	88.6 (85.8–91.2)	0.265**	89.1 (83.3–92.9)	86.8 (81.6–90.0)	0.470**
Viable CD19	×10⁶/L	10276 (9070–12183)	10962 (8538–13690)	0.575**	9272 (8758-12152)	9335 (8360–13283)	0.451**	8806 (7374–11663)	9394 (8007–12677)	0.591**
	%	99.3 (99.0–99.4)	98.9 (98.1–99.3)	0.055*	98.9 (98.1–99.1)	97.7 (96.8–98.7)	0.113*	97.2 (96.4–98.4)	97.2 (94.9–98.2)	0.644*
Viable CD16/56	×10⁶/L	3711 (2513–5206)	4091 (2706–6057)	0.547**	3930 (2613–4563)	4162 (2494–5519)	0.745**	3527 (2610–4535)	3923 (2580–5746)	0.604**
	%	97.2 (95.9–97.7)	91.9 (89.8–95.4)	**0.011***	95.4 (93.8–96.3)	89.9 (86.7–93.2)	**0.005***	90.2 (89.5–91.7)	85.7 (80.7–91.9)	0.280*

Viable CD3	×10^6/L	47262 (45244–54249)	34345 (27547–45465)	0.068**	45860 (40475-50631)	32518 (26965–45277)	0.086**	47745 (34514–49932)	32660 (26180–43133)	0.078**
	%	98.2 (97.0–98.9)	93.5 (89.5–96.3)	**0.001***	98.5 (97.9–99.0)	94.5 (88.7–97.2)	**< 0.001***	98.2 (98.0–98.8)	94.7 (88.8–96.5)	**< 0.001***
Viable CD3/ CD8	×10^6/L	15625 (12370–18769)	12927 (9537–15560)	0.213**	14743 (11810-16087)	12385 (9333–14752)	0.288**	13861 (9797–18301)	11645 (9651–14012)	0.247**
	%	99.0 (98.6–99.1)	96.1 (94.5–97.1)	**< 0.001***	99.3 (98.7–99.5)	96.6 (94.6–98.2)	**< 0.001***	98.4 (97.9–98.9)	96.5 (94.6–97.9)	**0.002***
Viable CD3/ CD4	×10^6/L	29311 (27659-40131)	21608 (16361–27692)	0.052**	28612 (24480–38021)	21404 (16696–26764)	0.064**	28016 (23515–36943)	21262 (16196–25116)	**0.028***
	%	98.1 (96.5–98.7)	92.6 (86.7–94.6)	**< 0.001***	98.8 (98.4–99.3)	94.6 (90.5–97.3)	**< 0.001***	98.2 (97.8–98.8)	94.5 (90.8–97.1)	**< 0.001***

Statistically significant differences ($p < 0.05$) are marked in bold; * Mann-Whitney test; ** *t*-test

dual-platform considers the percentage of CD34+ cells as a ratio of leukocytes or total nucleated cell concentration measured only on haematology analyser (19). The damage caused by cryopreservation usually results in the complete lysis of cells, which makes them undetectable on flow cytometry, Sometimes the percentage of post-thaw viability of the remaining cell could be high, even if many cells were lost. Therefore, the analysis of the absolute number of viable cells reflects more clearly the impact of cryopreservation and thawing on the cells in the PBSC graft. Furthermore, the absolute viable cell count is used to calculate the total number of cells in the leukapheresis products, as well as for assessing post-thaw cell recovery. The results of our study showed that the analysis of the absolute number of viable cells is more useful than determining only the percentage of viable cells for the assessment of graft quality.

Previous studies have shown that the storage time between cell collection and cryopreservation affects the viability and recovery of CD34+ and CD3+ cells in allogeneic leukapheresis products, and it could be expected that the cells that were stored for longer periods of time would have lower viability and poor tolerance to longer exposure to cryoprotectant (20). Our study included 20 allogeneic products received from the WMDA collection centre that were before the cryopreservation stored > 24 hours, and we examined whether storage time affects the stability of those samples compared to 10 allogeneic products collected in our Centre that were stored less than 24 hours before cryopreservation. Our results showed that the storage period before the cryopreservation did not affect the viability of allogeneic samples after thawing in three measurement time points because

there was no statistically significant difference in viable cell absolute counts, except for viable absolute CD3+CD4+ cells whose viability decreased after three hours of thawing ($p = 0.028$) (Table II).

The limitation of the study was that we analysed the cryovials as a surrogate of the contents of the bag, and that it was not certain that results were completely reflective of what was happening with the cells in the bag after thawing. Regarding the incubation temperature used in our study, some of the previously mentioned studies examined the stability of the samples at RT and 4 °C (13, 15, 21). In our study, we examined the post-thaw stability only at 4 °C because we noticed clot formation in random samples when they were stored at RT, which was also observed by Fritsch *et al.* who found clot formation in virtually all sample tubes 1 h post-thaw at RT (21).

CONCLUSIONS

The results of this prospective study showed that cryoprotectant DMSO had different effects on the viability and stability of various leukocyte subpopulations in cryopreserved PBSC samples after thawing. 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 PBSC products must be infused immediately after thawing in order to prevent the further loss of cell viability and preserve the quality of the graft.

Abbreviations, acronyms, symbols. – 7-AAD – 7-aminoactinomycin D, ANOVA – analysis of variance, COVID-19 – Coronavirus Disease 2019, DMSO – dimethyl sulfoxide, HSC – hematopoietic stem cell, ISHAGE – International Society of Hematotherapy and Graft Engineering, ISO – International Organization for Standardization, JACIE – Joint Accreditation Committee of the International Society for Cellular Therapy and the European Group for Blood and Marrow Transplantation, PBS – phosphate-buffered saline, PBSC – peripheral blood stem cell, RT – room temperature, UCB – umbilical cord blood, WBC – white blood cells, WMDA – World Marrow Donor Association.

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