

## Autologous peripheral blood progenitor cells cryopreserved with 5 and 10 percent dimethyl sulfoxide alone give comparable hematopoietic reconstitution after transplantation

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**BACKGROUND:** Previous in vitro studies have demonstrated decreased apoptosis and necrosis in peripheral blood progenitor cells (PBPCs) cryopreserved with 5 percent instead of 10 percent dimethyl sulfoxide (DMSO). This study was carried out to investigate whether these in vitro findings were supported by clinical data concerning hematopoietic engraftment after autologous stem cell transplantations with PBPCs cryopreserved with 5 and 10 percent DMSO.

**STUDY DESIGN AND METHODS:** During a 6-year period, 103 consecutive patients with newly diagnosed multiple myeloma (MM; n = 58) and lymphoma (n = 45) were transplanted with autologous PBPCs. Throughout the first part of the period cells were cryopreserved with 10 percent DMSO and later with 5 percent. A retrospective comparison was carried out of the clinical results for these two groups.

**RESULTS:** No significant difference in median time to neutrophil and platelet (PLT) engraftment was demonstrated for MM and lymphoma patients transplanted with PBPCs cryopreserved with 5 or 10 percent DMSO. Time until neutrophil counts of more than  $0.5 \times 10^9$  per L was 10 days both for the 5 and 10 percent MM groups and 12 days for both the 5 and the 10 percent lymphoma patients. Median time until stable PLT counts of more than  $20 \times 10^9$  per L was 11 days in all four groups. In addition, transfusion requirements and duration of days admitted to hospital did not differ between the groups.

**CONCLUSION:** The routines for cryopreservation of autografts vary considerably between transplantation centers, and this makes it difficult to compare different clinical studies. Our results suggest that cryopreservation with 5 percent DMSO alone followed by storage in nitrogen is a simple, highly standardized, and safe procedure for cryopreservation of autologous stem cell graft.

High-dose chemotherapy followed by transplantation of cryopreserved autologous stem cells is used in the treatment of several malignant disorders. The most commonly used cryoprotectant is dimethyl sulfoxide (DMSO) alone or in combination with hydroxyethyl starch (HES).<sup>1-3</sup> DMSO concentrations between 2.2 and 20 percent are used at different centers.<sup>1</sup> Thus, despite its widespread use in clinical practice, the optimal DMSO concentration for cryopreservation of autologous stem cell grafts remains to be established. A better standardization of the procedures for preparation of autografts is therefore required to allow comparison of the results from various transplantation centers with regard to hematopoietic and lymphoid reconstitution and also for the evaluation of therapeutic strategies aimed to reduce the risk of relapse from contaminating malignant cells in the autografts.

The most often used DMSO concentration is 10 percent.<sup>1</sup> It would be an advantage, however, to reduce this amount of DMSO, because infusion of thawed DMSO-

**ABBREVIATIONS:** ASCT = autologous stem cell transplantation; MM = multiple myeloma.

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containing autografts may result in severe adverse effects. Even though serious events are seldom, infusion of DMSO is usually an unpleasant experience for the patients. Adverse effects can be due to the DMSO itself, DMSO-induced histamine release, or cell debris and lysis products from granulocytes.<sup>4-6</sup> Most frequently, the severity of adverse effects depends on the DMSO dose administered<sup>7-9</sup> and the patient age.<sup>6</sup> The less the amount of infused DMSO, the fewer complications.<sup>1,10-13</sup>

DMSO is essential for cryopreservation of the stem cells, but is not needed after the cells are thawed. Therefore, it is desirable to keep the infused DMSO dose to a minimum. Reduction of adverse events can then be achieved by increasing the cell concentration and thereby reducing graft volume and the total amount of DMSO<sup>14-18</sup> or by infusing the autograft over longer time, if necessary several days.<sup>19</sup> Another alternative is washing away DMSO from the graft before stem cell infusion.<sup>20-24</sup> A final strategy is to use DMSO concentrations lower than 10 percent when freezing the peripheral blood progenitor cell (PBPC) products.<sup>10-12,25-28</sup> Previous *in vitro* studies at our institution have demonstrated that the use of 5 percent instead of 10 percent DMSO for cryopreservation of autografts results in better viability in addition to less apoptosis and necrosis of CD34+ cells.<sup>26-28</sup> We have also demonstrated that clonogenic cells as well as early hematopoietic progenitors are preserved better with 5 percent. Based on these *in vitro* studies, we have during the past 3 years used 5 percent DMSO instead of 10 percent for cryopreservation of autologous stem cell grafts. Our experience is reported in this article, and we conclude that the use of 5 and 10 percent DMSO results in comparable hematopoietic reconstitution after autotransplantation.

## MATERIALS AND METHODS

### Study design and patients

Approval from the regional ethical committee was obtained before the study was initiated. All the 103 patients gave written informed consent and were included in the autologous stem cell transplantation (ASCT) program. None of the 58 patients with multiple myeloma (MM), 13 patients with Hodgkin's lymphoma, and 32 patients with non-Hodgkin's lymphoma had received ASCT previously. In this nonrandomized study, all patients were transplanted consecutively with autografts cryopreserved with 10 percent DMSO until 2003. Thereafter, 5 percent DMSO was used as the sole cryoprotectant for myeloma patients, followed by lymphoma patients in 2004. As induction therapy, myeloma patients received three cycles in 4-week intervals with 0.4 mg per m<sup>2</sup> intravenous (IV) vincristine on Days 1 through 4, 9 mg per m<sup>2</sup> doxorubicin IV on Days 1 through 4, and 40 mg oral dexamethasone on Days 1 through 4, 9

through 12, and 16 through 20 (VAD) before 2003. From then on they were randomized to receive either three VAD cycles or two cycles with 1000 mg per m<sup>2</sup> cyclophosphamide IV on Day 1 and 40 mg oral dexamethasone on Days 1 through 4 and 9 through 12 (CyDex) with 4-week intervals. The majority of lymphoma patients received either 1) three cycles with CHOP consisting of 750 mg per m<sup>2</sup> cyclophosphamide IV, 50 mg per m<sup>2</sup> doxorubicin IV, 1.4 mg per m<sup>2</sup> vincristine IV (maximum 2 mg) all on Day 1, and 100 mg per day prednisone orally on Days 1 through 5 (CHOP); or 2) MIME consisting of IV infusions of 500 mg per m<sup>2</sup> mitoguanon on Days 1 and 15, 1000 mg per m<sup>2</sup> ifosfamide from Day 1 through Day 5, 30 mg per m<sup>2</sup> methotrexate on Day 3, 100 mg per m<sup>2</sup> etoposide from Day 1 through Day 3, and 400 mg mesna three times a day from Day 1 to Day 5 (MIME). Patients were mobilized by disease-specific chemotherapy (2 g/m<sup>2</sup> cyclophosphamide for myeloma and MIME for lymphoma patients) followed by 5 µg per kg granulocyte-colony-stimulating factor (G-CSF) once daily for MM patients and twice daily for lymphoma patients until harvestings were completed. High-dose therapy was 200 mg per m<sup>2</sup> melphalan on Day 1 for MM patients and BEAM for lymphoma patients (BEAM = 300 mg/m<sup>2</sup> IV carmustine on Day 1, 150 mg/m<sup>2</sup> etoposide twice daily from Day -7 through Day -4, 200 mg/m<sup>2</sup> cytarabine every 12 hours from Day -7 through Day -4, and 140 mg/m<sup>2</sup> melphalan on Day -3). Since 2001 ASCT with the originally harvested graft has been given to MM patients at relapse; therefore, hematologists have altered the guidelines for the recommended amount of infused CD34+ cells ( $2 \times 10^6$ - $4 \times 10^6$ /kg), to save up for later ASCTs. Analysis of age, percentage of patients older than 60 years, and sex distribution revealed no significant differences between the groups (Tables 1 and 2). Storage time was registered in days for all patients from stem cell collection to infusion.

In this single-institution study, all patients were treated according to the same guidelines throughout both study periods. The patients were all treated with protective isolation when the total blood neutrophil count was less than  $0.5 \times 10^9$  per L, and prophylactic antibiotic therapy was not used. The official guidelines for antibiotic treatment during febrile neutropenia and established infections, guidelines for platelet (PLT) and red blood cell (RBC) transfusions, and the use of hematopoietic growth factors were the same throughout the two study periods.

### Hematopoietic stem cell harvesting, cryopreservation, and infusion

Harvesting, cryopreservation, and thawing was performed as described in detail previously.<sup>26</sup> Upgraded software version 5.1 until 2004 and since then version 7.0 of a commercially available cell separator (COBE Spectra, Cobe Laboratories, Gloucester, UK) were used.

**TABLE 1. MM patients (n = 58) autografted with PBPCs cryopreserved in 5 and 10 percent DMSO\***

	5% DMSO	10% DMSO	p Value
<b>Patient characteristics</b>			
Number of patients	32	26	
Age (years)	57 (43-68)	56 (42-70)	NS
Percentage of patients older than 60 years	43.8	38.5	NS
Percentage female	34.7	38.5	NS
Number of apheresis sessions needed	1 (1-2)	1 (1-3)	NS
Storage time in days between harvest and autograft infusion	32 (18-154)	32 (18-131)	NS
Chemotherapy before stem cell harvesting	43.8% VAD, 56.2% CyDex	96.9% VAD, 3.1% CyDex	0.004
Number of chemotherapy cycles before high-dose therapy	4 (2-6)	4 (3-5)	NS
<b>Characteristics of autografts</b>			
Total number of infused CD34+ cells × 10 <sup>6</sup> /kg	3.83 (2.0-6.2)	4.45 (2.3-8.0)	0.032
CD34+ cell viability (%)	95 (65-99)	81 (20-94)	0.001
<b>Engraftment data†</b>			
Neutrophil count of at least 0.5 × 10 <sup>9</sup> /L (in days)	10 (9-13)	10 (9-13)	NS
PLT count of at least 20 × 10 <sup>9</sup> /L (in days)	11 (9-16)	11 (9-15)	NS
PLT count of at least 50 × 10 <sup>9</sup> /L (in days)	14 (10-21)	13 (10-18)	NS
Absolute peripheral blood lymphocyte count (ALC) at the time of neutrophil engraftment	0.1 (0.0-1.1)	0.1 (0.0-0.9)	NS
Percentage of patients with ALC ≥ 0.5 × 10 <sup>9</sup> /L	41.9	42.3	NS
Median ALC on Day 15 (×10 <sup>9</sup> /L)	0.4 (0.1-1.8)	0.4 (0.2-1.6)	NS
<b>Transfusion requirements and duration of hospitalization</b>			
Number of PLT transfusions	1 (0-5)	1 (0-7)	NS
Number of RBC transfusions	2 (0-8)	2 (0-6)	NS
Number of days of hospital stay after HSC infusion	17 (9-45)	14 (10-21)	NS

\* Data shown are median (range).

† p Values for engraftment data, transfusion requirements, and duration of hospitalization are calculated by linear univariate analysis with total CD34+ cell as a covariate.

CyDex = cyclophosphamide and dexamethasone; VAD = vincristine, doxorubicin, and dexamethasone; NS = not significant.

**TABLE 2. Lymphoma patients autografted with PBPCs cryopreserved in 5 and 10 percent DMSO\***

	5% DMSO	10% DMSO	p Value
<b>Patient characteristics</b>			
Number of patients	23	22	
Age (years)	50 (15-64)	46 (16-61)	NS
Percentage of patients older than 60 years	23.9	11.3	NS
Percentage female	52.2	45.5	NS
Number of apheresis sessions needed	1 (1-3)	1 (1-3)	NS
Percentage of patients who had relapsed disease	13	13.6	NS
Storage time in days between harvest and autograft infusion	33 (23-168)	32 (22-131)	NS
Number of chemotherapy cycles before high-dose therapy	9 (2-18)	10 (2-18)	NS
<b>Characteristics of autografts</b>			
Total number of infused CD34+ cells × 10 <sup>6</sup> /kg	4.34 (2.5-8.0)	4.38 (2.6-9.7)	NS
CD34+ cell viability (%)	95 (80-99)	84 (42-99)	0.001
<b>Engraftment data†</b>			
Neutrophil count of at least 0.5 × 10 <sup>9</sup> /L (in days)	12 (9-18)	12 (9-20)	NS
PLT count of at least 20 × 10 <sup>9</sup> /L (in days)	11 (8-17)	11 (8-19)	NS
PLT count of at least 50 × 10 <sup>9</sup> /L (in days)	15 (10-22)	14 (12-20)	NS
Absolute peripheral blood lymphocyte count (ALC) at the time of neutrophil engraftment	0.1 (0.0-1.0)	0.1 (0.0-0.9)	NS
Percentage of patients with ALC ≥ 0.5 × 10 <sup>9</sup> /L	69.6	65	NS
Median ALC at day 15 (×10 <sup>9</sup> /L)	0.5 (0.3-2.2)	0.5 (0.2-1.4)	NS
<b>Transfusion requirements and duration of hospitalization</b>			
Number of PLT transfusions	6 (2-22)	5 (1-15)	NS
Number of RBC transfusions	5 (2-12)	4 (2-8)	NS
Number of days of hospital stay after autograft infusion	19 (13-39)	18 (13-25)	NS

\* Data shown are median (range).

† p Values for engraftment data, transfusion requirements, and duration of hospitalization are calculated by linear univariate analysis with total CD34+ cell as a covariate.

NS = not significant.

Large-volume leukapheresis processing 4× the patient total blood volume was performed routinely. A combination of ACD-A (Baxter FA, Lessin, Belgium) at a blood-to-citrate ratio of 18:1, and 2500 IU of heparin (Leo Pharma AS, Oslo, Norway) was added to each 500 mL of ACD-A solution used for anticoagulation. Moreover, ACD equivalent to 10 percent of the estimated harvest volume was added initially to the collection bag.

The final mononuclear cell (MNC) concentration was  $0.75 \times 10^8$  to  $2.5 \times 10^8$  per mL. CD34+ cell quantification was carried out by flow cytometry with a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA) and its accompanying software (CellQuest, Becton Dickinson). PBPC bags and vials were stored in liquid nitrogen at  $-180^\circ\text{C}$  until stem cell infusion. After medication, autografts were infused through a line without filter, and infusions were completed within 10 minutes. A dose of 5  $\mu\text{g}$  per kg G-CSF was generally started 4 days after transplantation for MM patients and continued until neutrophil engraftment. Neutrophil engraftment was defined as the first of 2 subsequent days with neutrophil counts of more than  $0.5 \times 10^9$  per L and time until PLT engraftment as the first of 2 consecutive days with PLT counts of more than 20 (alternatively  $>50$ )  $\times 10^9$  per L without PLT transfusions. Duration of the hospital stay was calculated from the day of autograft infusion.

### Statistical analyses

The statistical analyses were performed with a standard software package (SPSS 14.0, SPSS, Chicago, IL). Most variables displayed a skewed distribution. The U test for two independent samples was utilized to calculate all p values. MM patients in the 5 percent DMSO group received significantly fewer CD34+ cells when they underwent ASCT than the patients in the 10 percent DMSO group ( $p = 0.032$ ). Therefore, we performed a univariate analysis with total number of CD34+ cells as a covariate in the MM group. p Values were considered as significant when less than 0.05.

## RESULTS

### Time until hematopoietic reconstitution

Median time to neutrophil engraftment with peripheral blood neutrophil counts of at least  $0.5 \times 10^9$  per L was 10 days for both MM groups and 12 days for both lymphoma groups (Tables 1 and 2). Median time to PLT engraftment with a PLT count exceeding  $20 \times 10^9$  per L was 11 days for all four groups (Tables 1 and 2). Of the MM patients, eight were discharged before they reached a peripheral blood PLT count of  $50 \times 10^9$  per L: six of them in the 5 percent MM group and two in the 10 percent MM group. Three lymphoma patients were

also discharged before they reached a PLT count of  $50 \times 10^9$  per L: two of them were in the 5 percent lymphoma group and one in the 10 percent lymphoma group. Median time to PLT engraftment with a PLT count exceeding  $50 \times 10^9$  per L was 1 day longer for both 5 percent MM and 5 percent lymphoma groups compared to the corresponding 10 percent groups: 14 days versus 13 days and 15 days versus 14 days, respectively. These differences were not significant, however.

The peripheral blood lymphocyte counts at the time of neutrophil engraftment (neutrophils,  $>0.5 \times 10^6/\text{L}$ ) did not differ between the groups (Tables 1 and 2). We also found no significant differences between the groups regarding absolute lymphocyte counts on Day 15.

### Infused CD34+ cells

All autografts were subject to short-time storage with the longest storage time being 6 months. Median storage time was 32 days for both 5 and 10 percent MM and 33 and 32 days for 5 and 10 percent lymphoma, respectively (Tables 1 and 2). The total number of transplanted CD34+ cells (both viable and apoptotic cells) was lower for the 5 percent MM group:  $3.8 \times 10^6$  per kg compared to  $4.5 \times 10^6$  per kg for the 10 percent MM group ( $p = 0.032$ ). This was due to the change in transplant guidelines when hematologists wanted to save up CD34+ cells for a later ASCT in case of relapse. The total number of infused CD34+ PBPCs, however, did not differ significantly between the two lymphoma groups (5 and 10% lymphoma). The CD34+ cell viability was significantly higher when the PBPCs were cryopreserved with 5 percent DMSO both for the myeloma (median values 95% vs. 84%;  $p = 0.001$ ) and for the lymphoma patients (95% vs. 81%,  $p = 0.001$ ). The number of infused viable CD34+ cells, however, did not differ between the two myeloma groups, while the 5 percent lymphoma group received more viable cells.

### Side effects, transfusion requirements, and duration of the hospital stay

No serious toxicities were registered during or immediately after stem cell infusions, the most common side effect being nausea (18 of 24 patients with side effects). The use of 5 percent instead of 10 percent DMSO was associated with a nonsignificant decrease in the percentage of patients with registered side effects; this was true for both myeloma (16% vs. 27%) and lymphoma patients (16% vs. 36%).

Lymphoma patients required more PLT and RBC transfusions than the MM patients, and this was independent of whether the PBPC product was cryopreserved with 5 or 10 percent DMSO (Tables 1 and 2). No significant differences were found, however, between 5 percent versus

10 percent MM and 5 percent versus 10 percent lymphoma patients regarding duration of the hospital stay (Tables 1 and 2).

## DISCUSSION

Cryoprotectants, cell concentration, and storage conditions including the storage temperatures are all important for maintaining hematopoietic progenitor cell functionality after freezing and thawing, especially when autografts are subject to long-term storage.<sup>15,16,26,27,29</sup> Storage in temperatures higher than  $-180^{\circ}\text{C}$  and/or DMSO concentrations lower than 10 percent is probably not disadvantageous, when products are used within few months. Cryopreserved stem cell grafts, however, show decreased MNC viability after 6 months of storage at  $-80^{\circ}\text{C}$ .<sup>11</sup> Even though the viability of CD34+ cells was not directly investigated in this study, one should be cautious when with a higher storage temperature.<sup>30</sup> Although our study is not randomized, the results strongly suggest that the hematopoietic engraftment potential of short-time-stored PBPCs cryopreserved with 5 percent DMSO is not inferior to products frozen with 10 percent DMSO.

It is well known that engraftment is faster for the patients who receive higher total prefreeze or higher viable CD34+ cell doses.<sup>3,12,16,29,31-33</sup> In our study, despite the fact that 5 percent MM patients were transplanted with fewer CD34+ cell doses, they did not show delayed hematologic engraftment. This observation further supports the fact that 5 percent DMSO is at least as good as 10 percent DMSO as the sole cryoprotectant in the ASCT setting.

The percentage of viable CD34+ cells was higher in autografts frozen with 5 percent DMSO, but this did not result in faster hematopoietic reconstitution. The present observations, however, are consistent with our earlier results<sup>34</sup> where we could not show any stronger correlation of engraftment kinetics with the number of infused viable CD34+ cells than with the total CD34+ cell number. In a recently published study, Hicks and coworkers<sup>35</sup> also showed no significant correlation between CD34+, viable CD34+, or viable CD34+/CD133+ cell counts with PLT engraftment in 20 MM patients, but they found significant correlation of CD34+/CD133+ cell counts with neutrophil engraftment. In contrast, other investigators have reported that postthaw viable CD34+ cell counts predicted hematopoietic engraftment.<sup>29</sup> A possible explanation for all these observations, including our observations of similar engraftment despite increased viability with 5 percent DMSO, could be that when the number of infused CD34+ cells is above a certain threshold, engraftment kinetics will be similar.<sup>12</sup>

To the best of our knowledge, there is only one previous study comparing the clinical use of 5 percent versus 10 percent DMSO alone for cryopreservation of

autografts.<sup>12</sup> These authors investigated effects of storage at  $-80^{\circ}\text{C}$  instead of storage in nitrogen as we did. They observed that 5 percent DMSO caused slower hematologic recovery compared to 10 percent, despite the fact that the former patient group received higher CD34+ cell doses ( $n = 251$  and  $n = 47$ , respectively). The differences in reconstitution, however, were not clinically significant for patients transplanted with at least  $1.5 \times 10^6$  per kg CD34+ cells. We observed no difference between our 5 and 10 percent DMSO groups. We may have missed detecting minor differences, however, especially when comparing frequencies of side effects that were low both when using 5 and 10 percent DMSO, due to our relatively small patient groups. Our findings are fully in agreement with the small study of Curcoy and colleagues<sup>25</sup> describing 13 patients with a median age of 11.8 years (range, 4.5-19.2 years) who underwent autologous transplantation with successful engraftment after short-time storage in 5 percent DMSO.

As Windrum and colleagues<sup>1</sup> have described, the used DMSO concentration shows a wide variation (from 2.2 to 20%) between transplant centers. DMSO is used either as the sole cryoprotectant or in combination with HES. There are only two hematopoietic engraftment studies comparing 10 percent versus 5 percent DMSO and storage in nitrogen,<sup>2,36</sup> and in both these studies 5 percent DMSO was used in combination with HES. The first study found no difference in granulocyte and PLT engraftment with 5 percent DMSO, while the second showed significantly faster neutrophil engraftment and equivalent PLT engraftment. Nevertheless, it is difficult to compare these results with ours due to their use of HES.

Time until posttransplant lymphoid reconstitution has been described as a prognostic variable in several malignancies, including MM and lymphoma.<sup>37-39</sup> This variable did not differ between our 5 and 10 percent groups either.

DMSO reduction strategies like washing of cells before infusion or with DMSO concentrations lower than 10 percent tend to reduce complication rates.<sup>1</sup> Thus, the overall available data strongly suggest that cryopreservation of autologous stem cell grafts with 5 percent instead of 10 percent DMSO will reduce the early complication rate without any adverse effect on time until reconstitution.

In conclusion, future clinical studies of autotransplantation in the treatment of malignant diseases should probably be based on a better standardization of the procedures for cryopreservation of the autografts. Our present results suggest that 5 percent DMSO alone should be used for cryopreservation. Further studies are needed, however, to clarify whether the engraftment potential of PBPCs cryopreserved with 5 percent DMSO is good enough also for long-time storage, for example, beyond 2 years.

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