Citation: Cellular Therapy and Transplantation, Vol. 1, No. 1, 28 May 2008, p. 35-42.
doi: 10.3205/ctt2008-05-28-002-en

Submitted: 10 October 2007, accepted: 19 May 2008, published: 28 May 2008

The influence of autologous marrow mesenchymal stem cell infusion on hematopoiesis reconstitution after hematopoietic stem cells autotransplantation in children with oncological and hematological diseases

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Abstract

Aim: This investigation was undertaken to study the possibility for the application of mesenchymal stem cells (MSCs) for hematopoiesis support and reduction of the neutropenia period after autologous HSCs transplantation for children with oncohematological disorders and graft insufficiency of CD34+ cells/kg. **Patients and methods:** 24 children, who after collection of hematopoietic stem cells (HSCs) had low numbers of CD34+ ($\leq$ 2,5 x10^6/kg) in autotransplant, were involved in our investigation. Autologous co-transplantation of MSCs was used for 7 adolescents; and 17 patients who were only given HSCs represented a control. The number of polychemotherapy cycles depended on the specific therapy response, relapse development, and the refractory to therapy, and varied from 4 to 10 cycles. MSCs were isolated from the bone marrow (BM) of patients up to 30–50 days before the autologous transplantation and expanded in vitro. CFU-F analysis was carried out for all patients. Statistical analysis was carried out with the help of STATISTICA 6.0 software. Difference reliability in groups during transplant parameters analysis was evaluated by the Mann-Whitney test, and the correlation degree between parameters by the Spearman test. **Results:** About 25 ± 6.9 ml of bone marrow was utilized in order to obtain MSCs. The CFU-F number was about 5.26 ± 0.6 colonies per 105 bone marrow mononuclear cells. The MSCs number had increased an average ~104 times after expansion in vitro for each patient. The data analysis revealed a statistically reliable dependence between the high-dose chemotherapy cycles number received by patients before bone marrow collection and MSCs growth time until a monolayer in primary culture ($r = 0.79$, $p = 0.03$) formed. The median number of MSCs reinfused into the patient was 0.6 (range 0.3–1.1) x 10^6 MSCs/kg in one hour after HSCs transplantation. In the case of co-transplantation, the MSCs neutrophil recovery > 500/µl was in 10 days (range, 9 to 11 days), $\geq$ 1000/µl in 11days (range, 10 to 13 days) compared to 13 days (range, 11 to 15 days), and 14 days (range, 13 to19 days) respectively, in the control group ($p = 0.002$ and $p = 0.001$ correspondingly). A reticulocyte number of $\geq$ 1‰ was observed by 10 days (range 9 to 12 days) and 14 days (range 11 to 17 days), respectively ($p = 0.004$). **Conclusion:** We determined an accelerated engraftment of HSCs transplant with low number CD34+ cells/kg in cases of autologous MSCs co-transplantation for children with malignant disorders. This approach is possible for children who have undergone prolonged myelotoxic and radiotherapy to expanse MSCs to efficient for co-transplantation volume.

Keywords: mesenchymal stem cells, colony forming unit-fibroblast, co-transplantation, hematopoietic engraftment

Introduction

Generally, the treatment of children with advanced stages or relapses of malignant disorders consists of high-dose chemotherapy, or, sometimes, radiotherapy with follow-up autologous transplantation of hematopoietic stem cells (HSCs). Multiple courses of intensive chemo and/or radiotherapy result in damage to the bone marrow cells involving all natural factors of antimicrobial defense, resulting in the patients‘ susceptibility to a wide spectrum of opportunistic infections. During the early post-transplant period, these infections and appropriate therapeutic approaches are determined by neutropenia. After transplant engraftment, the risk of severe bacterial infections becomes greatly reduced.

For most patients, engraftment of peripheral blood stem cell (PBSC) autotransplant occurs relatively early: average neutrophil counts of > 500/µl are observed by 11 days; increase in platelets to > 20 000 is reached by 12 days. However, for about 5% of the patients transplanted with autologous HSCs, a delayed reconstitution of neutrophils and platelets is observed, which is explained mostly by a low dosage of transplanted CD34+ cells per kg of patient‘s body weight [1, 2].

A highly attractive method of choice to shorten the period of hematopoietic reconstitution after transplantation of HSCs is being developed at present, i.e., a simultaneous autotransplantation of mesenchymal stem cells (MSCs).

List of abbreviations:

BM	bone marrow
BRCPON	Belarusian Research Centre for Pediatric Oncology and Hematology
CFU-F	colony forming units–fibroblasts
EDTA	ethylene diamine tetraacetate
FBS	fetal bovine serum
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hanks Balanced Salt Solution
HD	Hodgkin's disease
HSCs	hematopoietic stem cells
IL	interleukin
IMDM	Iscove Modified Dulbecco Medium
MNCs	mononuclear cells
MSCs	mesenchymal stem cells
PBSC	peripheral blood stem cell
SCF	stem cell factor
TGF	transforming growth factor

The main source of MSCs is bone marrow, which contains only 1-2 of these cells per 10000 of nucleated cells [3, 4, 5]. They have a fibroblast-like morphology and form specific fibroblast colony-forming units (CFU-F) when cultured in vitro. MSCs possess great proliferative capacity; and the cells obtained from young bone marrow donors are able to divide some 24-40 times, and to expand the cell population by up to 1 million times [6, 7, 8]. Phenotypic characterization of MSCs has shown that these cells are negative for hematopoietic markers (CD34, CD45, CD14, CD31, CD133), and positive for CD44, CD105 (SH2), CD166, CD73 (SH3), CD140 antigens [5, 9]. MSCs are multipotential progenitors and possess high plasticity. After cultivation in vitro and implantation, mesenchymal cells are able to differentiate into bone, cartilaginous, muscular, adipose tissues, and bone marrow stromal cells, as controlled by local factors and the microenvironment of the implantation area [5, 9, 10, 11, 12].

In such a way, MSCs give rise to bone marrow stromal cells that support hematopoiesis, by means of cytokine production (e.g., IL1, IL6, IL7, IL8, IL11, IL12, IL14), as well as Flt-3 ligand and SCF, which induces G-CSF and GM-CSF production [4, 9, 13]. Moreover, MSCs greatly support *in vitro* formation of megakaryocytes and platelet cells [14]. This work concerns the issues of hematopoiesis reconstitution in children with oncohematological disorders. Autologous transplantation of HSCs has been recommended for these patients, but after HSCs mobilization and collection the amounts of CD34+ cells per kg were not sufficient for fast engraftment. Co-transplantation with MSCs was applied for these patients.

Patients and methods

Patients: Twenty-four children were involved in our investigation. All of them were initially included in the protocol of treatment in BRCPOH and had neoplastic disorders at an advanced stage (II-IV), i.e., Hodgkin's disease (HD), non-Hodgkin's lymphomas and Ewing's sarcomas. Autologous transplantation of HSCs formed a part of the standard treatment protocols for these diseases. After collection of HSCs, we detected an insufficiency in all of these patients of CD34+ cells ($\leq$ 2,5 x10^6/kg) in transplants, due to either weak mobilization response induced by G-CSF, or excessive weight. The study was performed over a relatively long time period (June 2003 to May 2005). After obtaining written consent from the patients or their parents, autologous transplantation of MSCs was applied to seven adolescents, in addition to PBSC autotransplantation (five patients) and bone marrow autotransplantation (two cases), to investigate the influence of MSCs upon the rates of autotransplant engraftment. Eleven patients were auto-transplanted with PBSC (control group 1), and the six patients transplanted with bone marrow without MSCs autotransplantation were considered as control group 2.

Mobilization and collection of HSCs: PBSC mobilization in patients was carried out using G-CSF Granocyte (Sanofi – Aventis, France), according to the following schedule: G-CSF, 10 μg/kg within 24 hours subcutaneously for 5 to 10 days, depending on the body mass of the patients. The lymphocyte counts were observed on a daily basis. PBSCs were harvested when the lymphocyte numbers exceeded $\geq$ 25 x 106/ml. A blood cell separator "CS-3000+" (Baxter, USA) was used for stem cell apheresis.

The CD34+ cell numbers in the transplant were detected by flow cytometry (FACScan, Becton Dickinson, USA).

Mesenchymal stem cells: Up to 30–50 days before the planned PBSC auto-transplantation, 20–50ml portions of autologous bone marrow were taken under anesthesia via bone marrow puncture. Mononuclear cells were separated in Histopaque medium with a buoyant density of 1.077 (Sigma, USA), then twice washed in Hanks solution, re-suspended in Iscove's Modified Dulbecco Medium (IMDM) with 10% FBS (Sigma, USA), and transferred into 175cm^2 flasks filled with 30ml medium, at a concentration of 2-3 x 10^6/ml. The cells were incubated under 37^{o}C at 5% CO_2. Non-adherent cells were removed every 48 hours during the medium replacement. After reaching 80–90% confluence at the flask surface, the cells were detached with 0.25% trypsin-EDTA, and 1x10 cells were transferred into a new flask (passage 1). Several passages were carried out in a similar way. The *in vitro* expanded cells were identified by flow cytometry for the presence of typical MSC surface markers (CD105, CD90, CD140), and the absence of hematopoietic cells markers (CD34, CD45, CD14).

CFU-F analysis: To perform CFU-F counts, the mononuclear cells were isolated from bone marrow samples, and re-suspended in a full medium that contained IMDM supplemented with 15% FBS, L-glutamine, 2-mercaptoethanol and hydrocortisone (all reagents from Sigma, USA). Cell suspensions were then transferred into 60-mm Petri dishes. The cells were cultivated for 14 days (37^{o}C, 5% CO_2). After methanol fixation, the resulting colonies were stained according to Giemsa, and counted with inverted microscopy.

Statistical analysis: The data obtained was treated using STATISTICA 6.0 software. When analyzing the transplant parameters, the statistical significance of the differences between the groups was evaluated by the Mann-Whitney test. The degrees of correlations between the parameters were evaluated by the Spearman test.

Results

Patients' characteristics

In Table 1, we present the characteristics of seven patients involved in the experimental group who obtained additional infusions of MSCs. The age of these patients was 13 to 17 years; male/

Table 1. Characteristics of patients undergoing co-transplantation HSCs and MSCs

№	1	2	3	4	5	6	7
Age (years)	15	16	13	17	16	17	14
Sex	m	f	m	f	m	m	m
Diagnosis	HD	HD	Germinomas	Ewing's sarcoma	Ewing's sarcoma	NHL (B-type)	NHL (B-type)
State of disease	IV	II	IV	II	III	IV	IV
Relapse	early		early		early		early
Involvement of bone marrow	yes	no	no	no	no	no	no
Chemotherapy before THSC (number of blocs)	6	10	9	6	5	4	8
Prior radiotherapy	yes	no	no	yes	no	yes	no
Time THSC from start of disease (mo.)	13.5	10.5	13	7	8.5	7.5	12
Time THSC from mobilization (days)	215	33	70	80	93	138	7
Present disease status THSC	Stable	Refractory	Refractory	Remission	Refractory	Remission	Stable
Karnovsky index (%)	90	80	90	95	85	90	95

female ratio was 5:2. Two patients were diagnosed as Hodgkin's disease (HD), stages II and IV; two patients suffered with non-Hodgkin B-type lymphomas, stage III and IV, two of patients had Ewing's sarcoma, and one had forward mediastinal germinoma, IV stage. Treatment of all the patients started in accordance with the protocols approved in BRCPOH (for Hodgkin's disease, DAL HD 95, for Ewing's sarcoma, MMCU-99, for Non-Hodgkin's lymphoma, BFM-95 Rez, for germinoma, MAKEI-96). The numbers of chemotherapy cycles depended on the specific therapy response, relapse development, and the absence of a response to therapy; and varied from 4 to 10 cycles.

In addition to low CD34+ cell numbers in the transplant, the stabilization of the main disease in two weeks and Karnovsky Status >80% served as criteria for inclusion into this investigation. Two patients were in complete remission state, stabilization of the process was determined in two other patients, and there was no response to induction therapy in three adolescents.

Table 2. Characteristic of control group, undergoing autoHSCT

Characteristics		Number of patients
№ patients		17
Age (years)	7–18	
Sex	Male	12
	Female	5
Diagnosis	HD	8
	Ewing's sacroma	8
	NHD	1
Chemotherapy before THSC (number of cycles)		
Median (range)	6 (5–13)	
Prior radiotherapy	Yes	14
	No	3
BM involvement	Yes	2
	No	15

In patients with Hodgkin's disease, upon conditioning before autotransplantation of HSC, a high-dose polychemotherapy block BEAM was used (carmustin 300 mg/m^2+ Ara-C 800 mg/m^2+ etoposid 400 mg/m^2+ мelphalan 140 mg/m^2); in Ewing's sarcoma, TioBuM-140 (tiophosphamide 600 mg/m2+ busulphan 16 mg/kg + мelphalan 140 mg/m^2); with Non-Hodgkin's lymphomas BuVPEnd (busulphan 480 mg/m^2+ VP-16 900 mg/m^2+ endoxan 4500 mg/m^2), and in germinomas BuM-140 (busulphan 480 mg/m^2+ мelphalan 140 mg/m^2).

Table 2 shows the characteristics of the 17 patients involved in control group 1 (11 pts) and control group 2 (6 pts), who were subjected to autologous HSCT only.

Ex vivo MSCs expansion for autologous transplantation

For subsequent infusion together with autologous HSCs, the MSCs were cultured *in vitro* until the necessary amount had been obtained, depending on the body weight of each patient. Cells from passages 1 to 4 were used for autotransplantation in our patients. All mesenchymal cells obtained *in vitro* were morphologically homogeneous and exhibited a fibroblast-like shape in the monolayer at the surface of flasks. When re-suspended after trypsinization, they were large and rounded with a round nucleus, and they were three-fold larger than the neutrophils. (Table 3.)

The main parameters of growth dynamics for MSCs obtained from the patients' mononuclear cells *in vitro* are listed in Table 3. About 25.7 ± 2.8 ml of the bone marrow was utilized in order to obtain the MSCs. Of this volume, we yielded as many as 486.43 ± 108.61 x 10^6 mononuclear cells. A 90% confluence cell layer was obtained in the primary culture at about 17 ± 2 days of incubation. The cells were then detached from the flasks' surface and counted (first passage). An average of 17.14 ± 4.38 x 10^6 of MSCs were obtained after cell growth in primary culture.

After bone marrow collection, CFU-F analysis was carried out for all patients. This test allowed us to detect the relative number of MSCs (proliferative cells active in culture) among the nucleated cell fraction, isolated from patients' bone marrow probes. The CFU-F number was about 5.26 ± 0.6 colonies per 10^5 bone marrow mononuclear cells.

Table 3. Main growth parameters of mesenchymal stem cells (MSCs) obtained from patients mononuclear cells (MNCs) during *in vitro* expansion

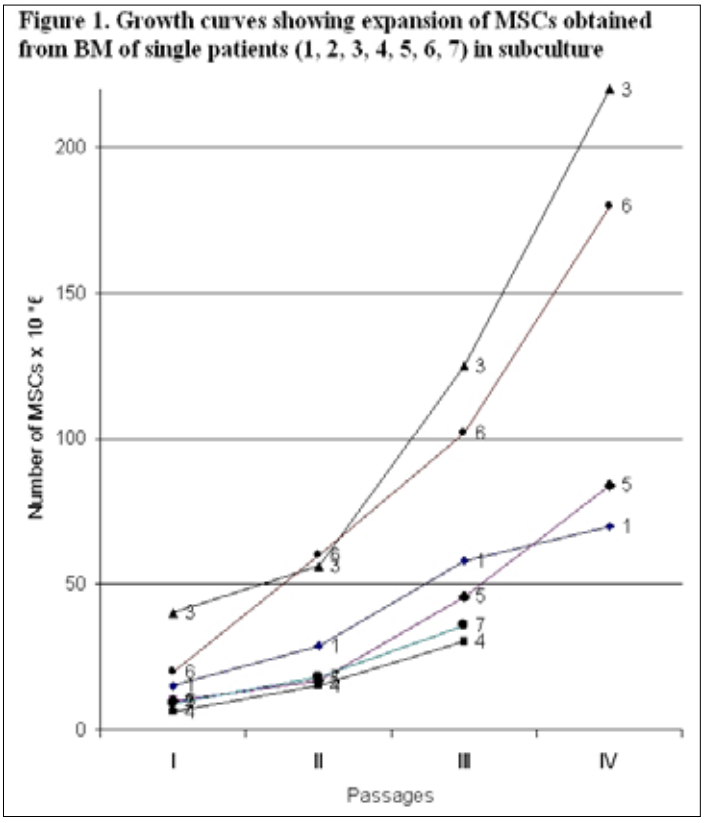
№	Volume of BM (ml)	Number of MNCs x10^6	Number of CFU-F per 10^5 MNCs	Number of MSCs after primary culture x10^6	Number of passages	Period of MSCs expansion (days)	Number of MSCs after expansion x 10^6
1	30	705	9	32	3	28	110
2	20	360	5.7	10	1	27	10
3	50	1260	6.5	40	5	47	220
4	20	140	1.5	6	3	35	34
5	20	500	4.5	10	4	44	84
6	20	320	5	20	4	30	180
7	20	120	4.6	3	4	47	40
Mean ± SD	25.7 ± 2.8	486.4 ± 108.6	5.3 ± 0.5	17.3 ± 4.0		36.9 ± 2.7	96.8 ± 21.2

Thus, the MSC number did increase ~10^3 times after incubation in the primary culture. After several passages were carried out, and the MSCs were incubated in subculture, their amount became ~10^4 times higher. Fig.1 represents the MSCs growth dynamics in subculture for each patient.

Fig.2 reflects the correlation between the numbers of initial MSCs in the bone marrow able to proliferate and to form colonies in culture (CFU-F number), and MSCs number obtained from the primary culture before they underwent subcultures ($r = 0.77$; $p = 0.04$).

Data analysis revealed a statistically reliable dependence between the high-dose chemotherapy cycles number received by patients before bone marrow collection and the time taken for MSCs growth until a monolayer in primary culture was formed ($r = 0.79$, $p = 0.03$).

Figure 1. Growth curves showing expansion of MSCs obtained from BM of single patients (1, 2, 3, 4, 5, 6, 7) in subculture



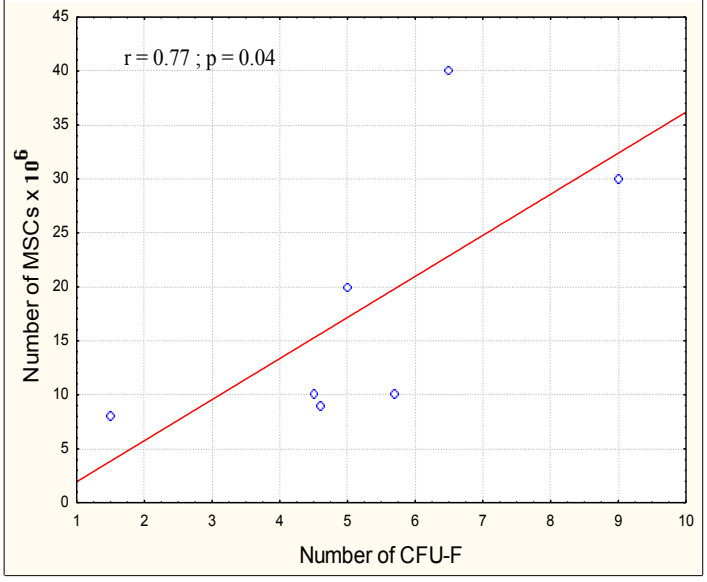
These results display the reduction of MSCs proliferative activity after chemo or radiotherapy.

HSCs and MSCs transplantation

Table 4 represents the HSCs and additional MSCs transplant characteristics for each patient. 5 children who had received PBSC as a transplant a median of 4.6 (range 3.0–6.9) x 10^8 /kg of nucleated cells were transfused including a median of 1.14 (range 0.64–1.3) x 10^6 /kg of CD34+ cells. Two patients who had received autologous bone marrow as a transplant 2.5 x 10^6 /kg and 8.1 x 10^6 /kg of nucleated cells were transfused including 1.0 x 10^6 /kg and 0.8 x 10^6 /kg of CD34+ cells, correspondingly. The median number of expanded MSCs reinfused into a patient was 0.6 (range 0.3–1.1) x 10^6 MSCs/kg in one hour after HSCs transplantation. Cells detached from the flasks' surface with the trypsine were washed twice in Hanks solution, counted and re-suspended in 20 ml of 0.9% NaCl solution. Intravenous injection of the cell suspension into the patient was performed within 5 minutes. The phenotype and sterility of the cell probes were analyzed.

Cells from passage 4 were used in 4 patients, cells from passage 2, in two patients. For one patient with IV stage HD, who had received 10 blocks of high-dose chemotherapy, MSCs from

Figure 2.



the first passage were used, in view of their weak proliferation activity, and, as a consequence, with prolonged growth in the main culture over 27 days, and attenuated cell proliferation after the first passage.

Hematopoietic engraftment after co-transplantation of HSCs and MSCs

Table 4 represents the results concerning the reconstitution of neutrophils and red blood cells after HSCs reinfusion for all patients. Five patients who received the MSCs infusion, in addition to autologous PBSC transplantation, and all patients in the control group had obtained 5 μg/kg of G-CSF, starting from the fifth day after transplantation until transplant engraftment. In the case of co-transplantation with MSCs, the median time of neutrophil recovery to ≥ 500/μl was on day 10 (range, 9 to 11), and ≥1000/μl was on day 11 (range, 10 to 13). These rates of neutrophil reconstitution are higher when compared to control group 1, where the median time of neutrophil recovery to ≥ 500/μl was on day 13 (range, 11 to 15), ≥ 1000/μl was on day 14 (range, 13 to 19).

Reconstitution of red blood cells was determined by the appearance of ≥ 1‰ of reticulocytes in the peripheral blood. This value was also higher in the experimental group with MSCs infusion compared to control group 1, and median red cells recovery was on day 10 (range 9 to 12) and on day 14 (range, 11 to 17) respectively.

In two children with co-transplantation of MSCs and autologous bone marrow, neutrophil recovery ≥ 500/μl in peripheral blood analysis was detected for both patients on day 14, ≥ 1000/μl on day 16 and day 14, the reticulocytes number ≥ 1‰ was on day 16 and on day 14, correspondingly. These rates of reconstitution are higher when compared to the autologous bone marrow transplantation in control group 2 (without MSCs infusion), where a median neutrophil recovery ≥ 500/μl was observed on day 24 (range 18 to 32), ≥ 1000/μl on day 25 (range 20 to 35) and median reticulocytes number ≥ 1‰ recovery was observed on day 26 (range 17 to 28).

Discussion

In our present work, we have for the first time proposed the viability of the curative potential for MSCs obtained from the bone marrow of children with oncohematological disorders, who have been pre-treated with high-dose polychemotherapy and radiotherapy. This investigation was undertaken in order to design a therapeutic strategy of MSCs application for hematopoietic support, and the reduction of the neutropenic period after an autologous transplantation of HSCs in children with insufficient amounts of CD34+ cells/kg.

The biological aspects of mesenchymal stem cells are still under intensive investigation. In spite of scarce experimental data, bone marrow-derived MSCs are increasingly being used in clinical settings. In most cases, either MSCs from healthy donors are cultured and expanded for use in allogenic co-transplantations with HSCs, or autologous MSCs are employed as implants for treatment of some non-oncological conditions, e.g., bone or cartilaginous disorders.

MSC‘s ability to support hematopoiesis *in vitro* has been shown experimentally by many workers, when co-culturing MSCs and HSCs. Mesenchymal stem cells are considered the precursors of stromal stem cells, osteoblasts, adipocytes, and endothelial cells that form areas of hematopoiesis-inducing microenvironment in bone marrow, thus supporting production of leucocytes, red blood cells and platelets [15, 16]. On one hand their effects upon hematopoietic precursors are exerted via secretion of soluble cytokines, chemokines, peptides, mediators and hormones and, on other hand, by formation of extracellular matrix from collagen, fibronectin and laminin molecules that provide homing and adhesion of hematopoietic cells. Interleukin-6 (IL-6), IL-1, IL-7, IL-8, SCF, Flt-3-ligand, colony-stimulating factors (CSFs), thrombopoietin, insulin-like growth factor, and transforming growth factor (TGF) are permanently synthesized by stromal cells, thus supporting the stability of blood cell counts within steady limits. IL-1 is the main inducer of cytokine production, whereas TGF is able to inhibit hematopoiesis [17].

Table 4. Hematopoietic reconstitution after simultaneous autotransplantation of HSCs and MSCs

Source of HSCs	Patients No	Graft characteristics			Neutrophil recovery (days)		Reticulocytes recovery ≥ 1‰ (days)
		Mononuclear cells (x10^8/kg)	CD34+ cells (x10^6/kg)	MSCs (x10^6/kg)	(≥500/ml)	(≥1000/ml)	
PBSC	1	4.6	1.3	0.6	10	11	10
	2	6.9	1.1	0.3	10	11	9
	3	5.7	0.64	1.1	11	11	11
	4	3.0	1.26	0.4	9	10	10
	6	3.4	0.76	1.0	11	13	12
	Med (min–max)	4.6 (3.0–6.9)	1.14 (0.64–1.3)	0.6 (0.3–1.1)	10 (9–11)	11 (10–13)	10 (9–12)
	Control 1 (n=11)	4.4 (1.82–8.2)	1.44 (1.1–1.8)		13 (11–15)	14 (13–19)	14 (11–17)
BM	5	2.5	1.0	1.0	14	16	16
	7	8.1	0.8	0.5	14	14	14
	Control 2 (n=6)	3.2 (1.6–7.1)	1.4 (1.3–2.3)		24 (18–32)	25 (20–35)	26 (17–28)

Some studies concerning the capacity of cultivated MSCs to support human hematopoiesis *in vitro* have revealed that, on contact with MSCs, both primitive hematopoietic precursors and committed cells are able to proliferate, thus maintaining their ability for self-replication and differentiation in renewing hematopoietic tissues [18]. The role of stromal cells is especially important due to their ability to prevent HSCs apoptosis [19, 20].

Graça Almeida-Porada et al, in their work on the influence of *in vitro* cultivated stromal cells on HSCs engraftment, used a xenogeneic model of prenatal transplantation of human cells into sheep embryos. They showed that in cases of human HSCs and MSCs co-transplantation, earlier and higher levels of donor cells in blood were attained. Moreover, HSCs engraftment was more effective with a combined treatment than HSCs transplantation alone [21]. Investigations of human hematopoietic cells from umbilical blood and MSCs co-transplantation into NOD/SCID mice confirmed the importance of MSCs in fast and stable hematopoietic cell engraftment decisively [22].

At the present time, limited data has been published concerning the application of hematopoietic and mesenchymal cell co-transplantation in clinics in order to shorten the period of neutropenia post-transplant in patients with malignant disorders. To our knowledge, no publications exist that concern the results of autologous MSCs utilization aiming to support fast and prolonged engraftment of HSCs auto-transplants in children with oncological and hematological diseases.

The issues of delayed reconstitution of granulocytes and platelets after HSCs autotransplantation still exist, however. A problem with autotransplant rejection still remains, and, in most cases, it occurs due to low doses of transplanted CD34+ cells/kg [1, 2]. At present, all studies on the application of autologous HSCs and MSCs co-transplantation within different groups of patients deal with high doses of CD34+ cells/kg in transplants: e.g., patients with breast tumors received an average of 13.9 x 10^6 CD34+ cells/kg, according to Koc O. et al (2000)[9]. For related allogenic PBSC transplantation to patients with malignant hematological diseases Lazarus HM et al (2005) employed a mean 5.0x10^6 CD34+ cells/kg [23]. And in the case of HSCs allogenic transplantation following T-cell depletion, D Cilloni et al (2000) transfused ≥ 2.2 x 10^6 CD34+ cells/kg [24]. However, numerous observations in large cohorts of patients with PBSC autotransplants suggest high levels of CD34+ in the transplant to be among the most important factors that affect neutrophil recovery after prescribed myeloablative therapy. A standard threshold dose of CD34+cells/kg weight for engraftment of autotransplant and hematopoiesis reconstitution is ≥ 2.5 x 10^6 cells/kg [25, 26, 27], and, at infusion doses over 5 x 10^6 CD34+cells/kg, the duration of cytopenia is noticeably reduced [28, 29, 30].

The main idea of our pilot study was to demonstrate the efficacy of supplementary autologous MSCs transplantation, in order to accelerate hematopoietic stem cells engraftment at low doses of CD34+ cells in transplants obtained by leukapheresis: i.e., 0.64–1.3 x 10^6 CD34+ cells/kg in PBSC autotransplant, and 0.8–1.0 x 10^6 CD34+ cells/kg in bone marrow autotransplants. An analysis of the resulting data revealed a significant reduction in the post-transplantation cytopenia period for the patients with MSCs co-transplantation after sub-optimal CD34+ cells mobilization, when compared to the control group. In particular, neutrophil reconstitution ≥ 500/μl was found at 9–11 days compared to 11–15, platelet increase ≥ 10000/μl, in 10–13 days as compared to 13–19 in controls, and red cells increased at 9–12 days against 11–17 days in the control group. In this case, it enabled us to avoid the commonly accepted procedure of repeated attempts at PBSC collection, or harvesting considerable bone marrow volumes in case of insufficient CD34+ cell numbers in grafts upon primary harvesting. This is important for the patients who have undergone multiple cycles of chemotherapy or high-dose radiotherapy before autotransplantation, because the negative influence of chemo and radiotherapy on CD34+ cell number is well documented. Bensinger et al (1994) have revealed that each cycle of chemotherapy results in reduction of CD34+ cell numbers by 0.2 x 10^6 cells during leukapheresis for patients without radiotherapy. Meanwhile, a round of radiotherapy results in CD34+ cells number reduction by 1.8 x 10^6 after PBSC collection [25].

A number of publications have demonstrated that bone marrow stroma suffers significantly as a result of high-dose chemotherapy or radiotherapy [31,32]. Stromal cells (4–5 week cultures) from the patients after conditioning with busulphan and cyclophosphamide are able to produce monolayers in only 20% of cases, in comparison with 80% in healthy donors [33], and it is not always possible to obtain enough MSCs *in vitro* for infusion into patients [24].

Our data also confirms a reduced MSCs proliferation capacity after chemotherapy or radiotherapy in our patients. This trend is supported by high correlation coefficient ($r = 0.79$, $p = 0.03$) between the numbers of previously received high-dose chemotherapy cycles, and MSCs expansion rate.

In our work we evaluated the functional state of stromal cells by their ability to proliferate in children with supplementary MSCs transplantation. To this purpose, CFU-F analysis was performed. The average CFU-F numbers in bone marrow of the patients was 5.26 ± 0.52 per 10^5 mononuclear cells. This number was considerably lower when compared to the results of CFU-F analysis in healthy donors [34]. In spite of this, the application of this MSC isolation method from bone marrow, and the technology of cell expansion considered by Koc O.[9], we expanded the primary amounts of MSCs by ~10^4 times. Thus we obtained sufficient amounts of MSCs within a mean of 36,6 ± 6,3 days after bone marrow aspiration.

Moreover, analysis of our data shows that MSCs infusion is already efficient at MSCs dose 0.3 x 10^6 cells/kg, thus being quite important in cases of MSCs expansion at limited times elapsing from MSCs aspiration to co-transplantation.

As a result of our pilot study, we confirmed that autologous MSCs co-transplantation may accelerate engraftment of HSCs transplants with low numbers of CD34+ cells/kg when treating children with malignancies. Expansion of MSC in sufficient amounts is an acceptable option in auto-transplants for children after they received prolonged myelotoxic therapy and radiotherapy.

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Ссылка: Клеточная терапия и трансплантация, том 1, номер 1, 28 мая 2008, с. 42,
doi: 10.3205/ctt2008-05-28-002-ru-a

Влияние инфузии аутологичных мезенхимных клеток костного мозга (МСК) на восстановление гемопоэза после аутотрансплантации гемопоэтических стволовых клеток у детей с онкологическими и гематологическими заболеваниями

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Резюме

Исследование предпринято, чтобы изучить возможности применения мезенхимальных стволовых клеток (МСК) для поддержки гемопоэза и сокращения периода нейтропении после аутологичной трансплантации гемопоэтических стволовых клеток (ГСК) детям с онкогематологическими заболеваниями и недостаточностью CD34+ клеток в трансплантате. В нашем исследовании участвовали 24 ребёнка, у которых после взятия гемопоэтических стволовых клеток (ГСК) было низкое содержание CD34+ клеток в аутотрансплантате (≤ 2 x10^6/кг). Аутологичная котрансплантация МСК применялась у 7 подростков, а в качестве контроля служили 17 пациентов, которым были введены только ГСК. Число циклов полихимиотерапии зависело от специфического ответа на терапию, развития рецидива, невосприимчивости к терапии, и варьировало от 4 до 10 циклов. МСК были изолированы из костного мозга (КМ) пациентов за 30-50 дней до аутологичной трансплантации и были размножены in vitro. Анализ CFU-F был выполнен для всех пациентов.

Статистический анализ был выполнен с помощью программного обеспечения STATISTICA 6.0. Достоверность различий результатов в группах при анализе параметров оценивали по тесту Mann-Whitney, и степень корреляции между параметрами – с помощью теста Spearman.

Результаты: В среднем 25 ± 6.9 мл костного мозга было использовано, чтобы получить МСК. Число CFU-F составляло 5.26 ± 0.6 колоний на 10^5 мононуклеарных клеток костного мозга. Число МСК для каждого пациента возрастало в среднем в 10^4 раз(а) после размножения in vitro. Анализ данных показал статистически достоверную зависимость между числом циклов высокодозной химиотерапии, полученной пациентами до сбора клеток костного мозга и сроками роста МСК, до формирования монослоя в первичной культуре ($r = 0.9$, $p = 0.03$).

Среднее число МСК, введенных больным, было равно 0.6 (от 0.3 до - 1.1) x 10^6 МСК /кг в течение 1 часа после трансплантации ГСК. В случае котрансплантации МСК, раннее восстановление нейтрофилов происходило через 10 дней (от 9 до 11 дней), по сравнению с 13 днями (11 - 15 дней) в группе контроля ($p = 0.002$). Повышение доли ретикулоцитов свыше ≥ 1 ‰ наблюдалось, соответственно, через 10 дней (от 9 до 12 дней) при ТМСК и 14 дней в контроле (от 11 до 17 дней, $p = 0.004$).

Заключение: Выявлено ускоренное приживление трансплантанта ГСК при низком числе CD34+ клеток/кг в случае аутологичной (совместной ко-трансплантации МСК у детей со злокачественными заболеваниями. Эта возможность имеется для детей, которые подверглись продолжительной миелотоксической и радиационной терапии для увеличения количества МСК до оптимального для совместной трансплантации.

Ключевые слова: мезенхимальные стволовые клетки, колониеобразующие единицы фибробластов (КОЕ-Ф), совместная трансплантация, гемопоэтическое приживление