MOLECULAR AND EXPERIMENTAL BIOLOGY IN MEDICINE

THE ASSOCIATION OF VIABLE MONONUCLEAR CELLS AND CFU-GM WITH TIME OF HEMATOPOIETIC ENGRAFTMENT

Ana Livun, Katarina Marija Tupek

Abstract: The quality of the collected hematopoietic stem cells (HSC) product is of primary importance for each patient in the transplantation process. We have collected data on the number and viability of cells in the HSC engraftment, both before and after freezing, and compared it to the rate of hematologic recovery to evaluate and to determine the laboratory parameter that sufficiently determines the rate of recovery after transplantation. We concluded that the average percentage of viability and the average stem cell count per kg of patient was consistent with the recommended values; the number of CFU-GM in the fresh leukapheric sample was associated with the rate of neutrophil recovery, whereas no statistical significance was found in association with other measurements, and the cell viability and growth of CFU-GM in this study did not show a direct impact on patient recovery rate.

Clinical Department of Laboratory Diagnostics, University Hospital Dubrava, Zagreb, Croatia

Corresponding author:
Ana Livun
Clinical Department of Laboratory Diagnostics, University Hospital Dubrava, Zagreb, Croatia
e-mail: alivun@kbd.hr

Submitted: June, 2018
Accepted: July, 2018

Key words: hematopoietic stem cell transplantation, CFU-GM colonies, hematologic recovery

INTRODUCTION

The hematopoietic stem cell (HSC) was the first type of stem cell to be discovered in humans, and so far, it is the only one that is used in routine clinical work.[1] This pluripotent HSC differentiates into immature cells of the myelopoietic and lymphopoietic systems where, by the process of proliferation and differentiation, they are transformed into completely functional, mature blood cells.[2] The HSC is primarily located in the bone marrow (BM), where the specific hematopoietic microenvironment regulates its functions.[3] Differentiation of the HSC depends on the presence of growth factors. Regardless of its primary location, the HSC shows significant mobility. In response to specific signals, the HSC may lose adhesion molecules and exit from the BM by the mobilization process or reintroduce itself into the BM.[4] This mobility makes HSCs the perfect candidates for the successful transplantation and treatment of innate and acquired malignant diseases of the blood and immune systems, as well as some solid tumors.

Today, three sources of HSCs are used for transplantation. These include the bone marrow (BM), the peripheral blood (PB) and the cord blood. Although the BM function can be restored after transplantation from all three sources, it is known that there are quantitative and qualitative differences between transplants.[5] Today, the traditional source of HSCs is slowly being replaced by a leucocyte concentrate from the PB by means of leukapheresis. This procedure is safer and simpler than BM extraction. The advantage of collecting HSCs from the PB is that the number of HSCs in the PB can be increased *in vivo* by the mobilization process using myelosuppressive doses of chemotherapy or growth factors.[6, 7] A leucocyte concentrate also contains myeloid cells, so the recovery of the BM function is faster after the transplantation of HSCs from the leukocyte concentrate than from the BM.

In clinical practice filgastrim, a human G-CSF (granulocyte colony stimulating factor) produced by recombinant DNA technology is used for the mobilization process. It increases the number of CD34+ cells 10 to 100 times. The synergistic effect of combined chemotherapy and G-CSF increases the number by 100 to 500 times.[8] Nevertheless, there is still a significant portion of patients who cannot collect enough HSCs. Patients (20-40%) are considered poor mobilizers if the number of CD34+ cells in the PB is less than $20x10^6$/L.[8] It is then possible to apply the following procedures: re-mobilization with the same or more intensive protocol, high blood volume leukapheresis and the introduction of a new mobilization agent.

After each leukapheresis procedure, the number of CD34+ cells is determined. Leukapheresis continues on a daily basis until the targeted cell count is collected. Usually, the required number of HSCs is collected from 1-3 leukaphereses. To determine when to start leukapheresis, daily monitoring of leukocytes and CD34+ cells in the PB is required. Leukapheresis uses a cell separator that identifies target cells by blood centrifugation due to the specific density of the blood cells.[9] The total blood volume of the patient, which is 8-12 L, is treated 2-3 times, and the procedure lasts for 3-4 hours.[8]

The goals of HSC transplantation are the complete restoration of stem cells in the BM as well as the fastest possible hematological recovery of neutrophils and platelets, with fewer side effects and treatment costs. The quality of the collected HSC product is of primary importance for each patient. After completing the leukapheresis procedure, the quality of the collected cells is always analyzed. For now, there is no generally accepted method of analyzing the primitive stem cell; however, in practice, two empirically identified parameters are used: the number of CD34+ cells and the number of colonies in a short-term cell culture. These indicators are expressed as the number of cells per kilogram of body weight. Also, the safety of the leukapheresis product, with regards to possible microbiological contamination, is very important.

The aim of this research was:

1) to collect data - the number and viability of cells in the HSC engraftment, both before and after freezing,

2) to compare this data from all the patients treated at the Department of Hematology since 2015 with the rate of hematologic recovery,

3) based on the results obtained, to evaluate and to determine the laboratory parameter that sufficiently determines the rate of recovery of patients after transplantation.

MATERIAL AND METHODS

From November 6th, 2015 to September 29th, 2016, leukocyte concentrate samples from 25 patients were processed. All the patients were treated in the Department of Hematology, University Hospital Dubrava, and have received their HSC engraftment. After transplantation, they were monitored for up to 4 weeks. Monitoring means checking their complete blood count every day after the transplantation in Clinical Department of Laboratory Diagnostics, University Hospital Dubrava on the hematologic analyzer LH750 Beckman Coulter, USA.

The study included 25 patients (8 female and 17 male). The average age was 52 (range: 22 to 68). Patients had three types of diagnosis, multiple myeloma (MM) - 10 patients, non-Hodgkin's lymphoma (NHL) - 14 patients and Hodgkin's disease (MH, from Morbus Hodgkin) - 1 patient.

The patient leukocyte concentrate samples were divided into three groups:

1) fresh samples - samples separated from leukocyte concentrates immediately after leukapheresis,

2) control samples - samples to which cryoprotectives have been added after leukapheresis and then stored in liquid nitrogen for at least 48 hours (these samples give a realistic assessment of the quality of the frozen leucocyte concentrate), and

3) control samples of transplants - samples extracted from the transplantation dose immediately before transplantation.

We determined the viability and established a short-term stem cell culture for all three groups.

Viability was determined by dividing the leukocyte concentrate samples in an equal ratio of Trypan blue solution (Figure 1). Fresh samples were expected to have a viability of 90-100% while in the two other groups that were frozen, the viability was expected to be lower. There was no cut off value for control samples in this research.

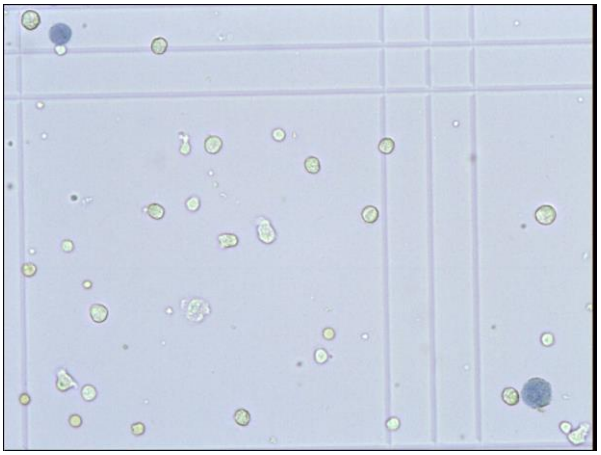

Figure 1. An example of cell viability. The viability is 90% (there are 18 live cells and two dead cells in the field).

For short-term stem cell culture, we used Methocult H4433 and a semi-solid medium. Cells were cultured for 14 days at 37°C with 5% CO_2 in an incubator, and granulocyte-macrophage colonies were counted with an inverted microscope. The counted colonies were

expressed as the number of colonies x10^5/MNC (Figure 2).
The rate of hematologic recovery was collected at 3 points for leukocytes and 2 points for neutrophils. We monitored the complete blood count daily, and noted when the number of leukocytes in the peripheral blood reached >0.5; >1 and >5x10^9/L. Hematologic reconstitution considered for neutrophils was >0.2 and >0.5x10^9/L.

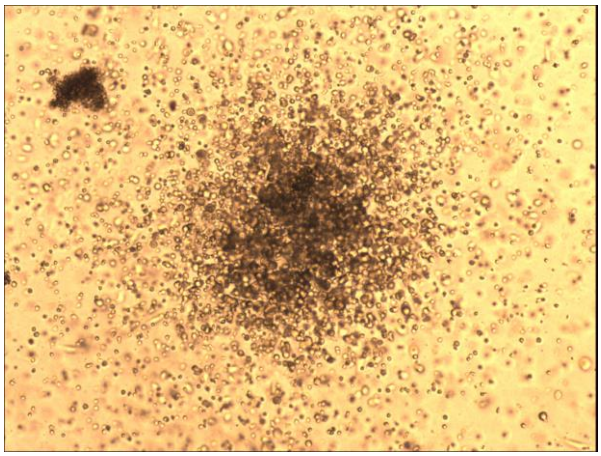

Figure 2. An example of a CFU-GM (Colony Forming Units-Granulocyte Macrophage) colony after 14 days of incubation.

The statistical data included descriptive statistics from which the median values and ranges (minimum and maximum values) were calculated for all of the processed numerical data, while the qualitative data is shown in absolute (N) and relative numbers (%). A graphical presentation of the recovery rate was formulated in the form of Kaplan-Meier curves, whereby patients who did not reach the required concentration of cells were excluded. The correlation between the rate of cell recovery with the graft quality was examined by the Cox regression test, with P <0.05 as a measure of statistical significance. The association was expressed by the risk ratio and the limits of its 95% significance. MedCalc (Version 9.2, MedCalc Inc., Mariakerke, Belgium) was used for the statistical analysis.

RESULTS

The study included 25 patients. One patient died of treatment complications and its data have been excluded from this research since the hematologic reconstitution was not achieved.
Viability of fresh samples were high as expected and other values are listed in Table 1 with the numbers of CFU-GM colonies. CFU-GM colonies were developed from all the samples.
According to the CFU-GM data from Table 1 and the patient mass data, the average amount of stem cells per kilogram of body weight was calculated to be 11.1 (3.6-35.8) x10^6/kg.[15]

Table 1. The percentages of viability and the number of CFU-GM colonies in 3 different samples

SAMPLE	VIABILITY (%)	CFU-GM (Nx10^5/MNC)
leukapheresis	99 (94 -100)	56 (28-200)
control	78 (48 - 99)	42 (0-200)
transplant	81 (38-99)	49 (17-132)

Legend CFU-GM - Colony Forming Units-Granulocyte Macrophage; MNC - mononuclear cell

Table 2 shows how many days it took for the post-transplantation in the PB of patients to reach the default value of leukocytes and neutrophils and the dynamics are shown in Figure 3 for leukocyte recovery and Figure 4 for neutrophils.

Table 2. The recovery rate of patients after transplantation

	NO. OF LEUKOCYTES IN PB (10^9/L)	RECOVERY RATE (days)
leukocytes	> 0.5	10 (9 - 11)
	> 1.0	11 (9 - 12)
	> 5.0	13 (10 - 22)
neutrophils	> 0.2	10 (8 - 10)
	> 0.5	10 (9 - 11)

Legend: PB - peripheral blood

The rate of leukocyte recovery (LCT) in the PB of patients was measured in days reaching the cell concentration of >0.5x10^9/L; >1x10^9/L and >5x10^9/L. The average recovery rate is 10 (9-11) days; 11 (9-12) days and 13 (10-22) days, respectively.
The correlation between the quality parameters (the number of stem cells and viability) with the patient's recovery rate is shown in Table 3. The table shows that only the concentration of stem cells in the fresh leukapheresis samples is significantly related to the neutrophil recovery rate measured to a concentration of >0.5x10^9/L (P=0.045), while all other values are not statistically significant (P>0.05).
This means that a significantly faster recovery of the neutrophil granulocytes up to a concentration of >0.5x10^9/L is expected with a higher amount of CFU-GM in a fresh sample, immediately after leukapheresis. The rate of recovery of other measured cells, as well as the concentration, does not depend on the number of stem cells, nor on the viability of mononuclear cells.

DISCUSSION

This research was conducted to assess how the quality of the engraftment affects the patient's recovery speed.

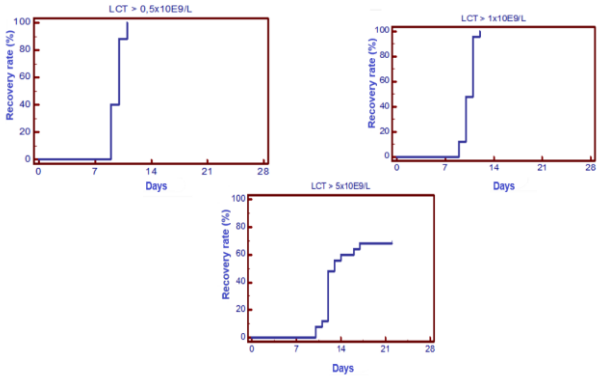

Figure 3. The rate of leukocyte (LCT) recovery in peripheral blood of patients measured in days reaching the cell concentration of >0.5x10^9/L; >1x10^9/L and >5x10^9/L.

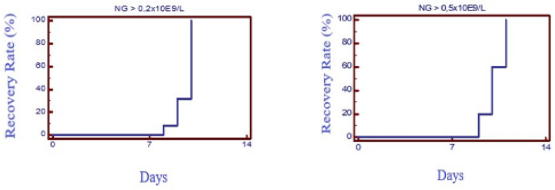

Figure 4. The rate of recovery of neutrophils (NG) in peripheral blood of patients measured in days reaching the cell concentration of >0.2x10^9/L and >0.5x10^9/L. The average recovery rate is 10 (8-10 for >0.2x10^9/L and 9-11 for >0.5x10^9/L) days.

Cross-quality was monitored through two parameters, cell viability, and the growth of CFU-GM colonies in three types of samples: leukapheresis samples, control samples and transplantation samples. In fresh samples the percentage of viability was very high, 99% (recommended value: >95%[14]), which is important since viability is the first indicator of biological activity in the cells that play a key role in BM recovery. In control samples and reinfusion samples the percentage was slightly smaller, 78% in the control samples and 81% in the transplanted samples (recommended value: >70%[14]). These results are very satisfactory and at the same time represent evidence of excellent survival of the cells after freezing. It is known that cell viability and even granulocyte recovery are reduced by freezing and thawing, which indicates that cryopreservation is an important factor when planning to introduce an HSC transplantation procedure.[10] The finding is consistent with the literature proving that postransplant engraftment is dependent on the quantity of hematopoietic cells with repopulating ability.[14]

Recent research suggests that the collection, manipulation, storage, and dehydration of the engraftment require a special approach as they may affect the overall transplantation performance.[10, 11] That is why new methods of storing samples, such as storage in a double bag and cryogenic chambers, are being developed, which affects cell viability and neutrophil recovery.[11]

After fourteen days we counted the colonies in the samples in which we had previously determined the viability. In the leukapheresis samples the largest growth of colonies, 56x10^5/MNC, was expected due to the apparent influence of the freezing and thawing process. In the control samples we counted 42x10^5/MNC, and in the reinfusion samples 49x10^5/MNC. According to this data (Table 1) and the patient weight data, the average number of stem cells per kilogram of body mass was reported[15] and, in this study, was equal to 11.1 (3.6-35.8) x10^6/kg, which is satisfactory. The target cell dose for collection and transplantation was 2×10^6 CD34+ cells/kg. Studies have shown that CFU-GM progenitor cells and mononuclear cells are the most important factors of neutrophil recovery, which subsequently suggests the recovery of the patient after reinfusion.[12, 13] This study also confirmed that the number of hematopoietic stem cells estimated by CFU-GM assay is a good and reliable routine test for prediction of hematopoietic recovery, as suggested before.[16]

Therefore, the recovery was also followed by two parameters: the leukocyte concentration in the PB and the concentration of the neutrophils in the PB. The first leukocyte growth (LCT>0.5x10^9/L) was recorded on the 10th day. As early as the next day, patients had LCT>1.0x10^9/L. The most significant increase in the leukocyte concentrations (LCT>5.0x10^9/L) in most patients occurred on the 13th day after reinfusion. The neutrophil recovery was monitored through two concentrations, NG>0.2x10^9/L and NG>0.5x10^9/L. In the majority of patients, on the 10th day there was an increase above the given concentrations. However, by further examination of the recovery rate with the cross-sectional quality indicators, only the stem cell concentrations in the fresh leukapheric samples were significantly associated with the neutrophil recovery rate measured to >0.5x10^9/L (P=0.045, Table 2), while all other values were not statistically significant (P>0.05). Specifically, the significantly faster recovery of the neutrophil granulocytes to a concentration greater than 0.5x10^9/L is expected with a higher number of CFU-GMs in the fresh sample, immediately after leukapheresis. Rowley's results support the findings of our study. As stated before, no relation was evident between the CFU-GM and time for recovery of peripheral blood cells.[17]

This research demonstrated that the cell viability and CFU-GM growth in cell culture, although important as benchmark parameters, do not have a direct impact on the recovery rate of our patients. From this we conclude that the quality was satisfactory, but did not have a direct impact on the speed of patient recovery for undefined reasons. In addition to the previously mentioned manipulation procedures that directly affect the quality of the transplant, it has been shown that the rate of recovery of patients after reinfusion depends on various factors such as age, diagnosis, treatment procedures, length of the disease and febrile post-reinfections.[18]

In a future extension of this study, both CFU-GM viability and CD34+ contents will be evaluated and compared with the data of illness characteristics.

Table 3. Correlation between the quality parameters*. The rate of leukocyte and neutrophil recovery for a total of five cell concentrations was compared to the number of CFU-GM and cell viability in the leukapheresis samples and control samples, and was expressed as a risk ratio (OR) and its 95% confidence limits (G95% OR).

Recovery Rate	Indicator	P	OR	G95% OR
LCT > $0.5x10^9$/L	*CFU-GM leukapheresis*	0.604	0.9921	0.9632 - 1.0220
	CFU-GM control	0.438	1.0115	0.9829 - 1.0409
	viability leukapheresis	0.381	1.4879	0.6141 - 3.6050
	viability control	0.641	0.9889	0.9436 - 1.0363
LCT > $1x10^9$/L	*CFU-GM leukapheresis*	0.164	1.0185	0.9927 - 1.0451
	CFU-GM control	0.483	0.9918	0.9692 - 1.0148
	viability leukapheresis	0.506	0.8906	0.6337 - 1.2516
	viability control	0.466	1.0133	0.9782 - 1.0496
LCT > $5x10^9$/L	*CFU-GM leukapheresis*	0.118	1.0214	0.9947 - 1.0489
	CFU-GM control	0.197	0.9843	0.9610 - 1.0082
	viability leukapheresis	0.314	0.8382	0.5952 - 1.1805
	viability control	0.410	1.0141	0.9810 - 1.0483
NG > $0.2x10^9$/L	*CFU-GM leukapheresis*	0.132	1.0215	0.9937 - 1.0501
	CFU-GM control	0.151	0.9813	0.9564 - 1.0068
	viability leukapheresis	0.661	1.0757	0.7776 - 1.4879
	viability control	0.322	1.0182	0.9827 - 1.0549
NG > $0.5x10^9$/L	*CFU-GM leukapheresis*	0.045	1.0272	0.1001 - 1.0562
	CFU-GM control	0.144	0.9817	0.9577 - 1.0062
	viability leukapheresis	0.806	0.9567	0.6729 - 1.3601
	viability control	0.424	1.0152	0.9785 - 1.0533

Legend: LCT – leukocytes, NG – neutrophils; CFU-GM - Colony Forming Units-Granulocyte Macrophage

CONCLUSIONS

The average percentage of viability was consistent with the recommended values (leukapheresis samples 99%, control samples 78% and 81% in transplanted samples);
The average stem cell count per kilogram of patient mass was also within the recommended values ($11.1x10^6$/kg);
The number of CFU-GM in the fresh leukapheric sample is associated with the rate of neutrophil recovery to a concentration >$0.5x10^9$/L, whereas no statistical significance was found in association with other measurements;
Cell viability and growth of CFU-GM are important parameters for the quality of the cut-off but in this study, they did not show a direct impact on patient recovery rate;
The data on cell viability and the increase in CFU-GM data obtained during this research fully satisfy the required criteria of good laboratory practice.

ACKNOWLEDGEMENTS

The authors would like to express their very great appreciation to Prof. Mladen Petrovecki. His willingness to give his time so generously has been very much appreciated.

REFERENCES

1. Bryder D, Rossi DJ, Weissman IL. Hematopoetic stem cells: The paradigmatic tissue specific stem cell. Am J Pathol. 2006;169:338-346.
2. Smith A. A glossary for stem-cell biology. Nature. 2006;441:1060.
3. Yin T, Li L. The stem cell niches in bone. J Clin Invest. 2006;116:1195-201.
4. Morrison SJ, Spradling AC. Stem cells and niches: mechanisms that promote stem cell maintenance throught life. Cell. 2008;132:598-611.
5. Gratwohl A, Baldomero H, Frauendorfer K, et al. Results of the EBMT activity survey 2006 on haematopoietic stem cell transplantation: focus on the use of cord blood products. Bone Marrow Transplant. 2008;41:687-705.

6. Socinski MA, Cannistra SA, Elias A, Antman KH, Schnipper L, Griffin JD. Granulocyte-macrophage colony stimulating factor expands the circulating hematopoietic progenitor cell compartment in humans. Lancet. 1989;1:1194-1198.
7. To LB, Shepperd KM, Haylock DN, Dyson PG, Charles P, Thorp DL, Dale BM, Dart GW, Roberts MM, Sage RE. Single high doses of cyclophosphamide enable the collection of high numbers of hemopoietic stem cells from the peripheral blood. Exp Hematol. 1990;18:442-447.
8. Reik RA, Noto TA, Fernandez HF. Safety of large-volume leukapheresis for collection of peripheral blood progenitor cells. J Clin Apheresis.1997;12:10-13.
9. Kessinger A, McMannis J. Practical considerations of apheresis in peripheral blood stem cell transplantation. Lakewood: COBE BCT. 1994, 25-34.
10. Majado MJ, Salgado-Cecilia G, Blanquer M, Funes C, González-García C, Insausti CL, Parrado A, Morales A, Minguela A, Moraleda JM. Cryopreservation impact on blood progenitor cells: influence of diagnoses, mobilization treatments, and cell concentration. Transfusion. 2011;51(4):799-807.
11. Abbruzzese L, Michieli M, Rupolo M, Toffola RT, Da Ponte A, Rossi FM, Lorenzon D, Simonelli C, Gattei V, De Marco L, Mazzucato M. A new freezing and storage procedure improves safety and viability of haematopoietic stem cells and neutrophil engraftment: a single institution experience. Vox Sanguinus. 2010;98(2):172-180.
12. Lewis A. Autologous stem cell derived from the peripheral blood compared to standard bone marrow transplant; time to engraftment: a sistematic review. Int J Nurs Stud. 2005;42(5):589-596.
13. Kozlowska- Skrzypczak M, Gil L, Komarnicki M. Factors affecting neutrophil recovery after autologous bone marrow-derived stem cell transplantation in patients with acute myeloid leukemia. Transplant Proc. 2009;41(9):3868-3672.
14. FACT-JACIE International Standards for Hematopoietic Cellular Therapy Product Collection, Processing, and Administration. 2015.
15. Ali MY, Oyama Y, Monreal J, Winter JN, Tallman MS, Williams SF, Singhal S, Gordon LI, Mehta J. Ideal or actual body weight to calculate CD34+ cell doses for autologous hematopoietic stem cell transplantation? Marrow Transplantation. 2003;31:861-864.
16. Petrovečki M, Nemet D, Kolevska T, Marušić M. Granulocyte-monocyte colony forming unit content of autologous bone marrow transplants in patients with haematological malignancy. Eur J Clin Chem Clin Biochem. 1995;33:687-691.
17. Rowley SD, Zuehlsdorf M, Braine HG, Colvin OM, Davis J, Jones RJ, Saral R, Sensenbrenner LL, Yeager A, Santos GW. CFU-GM content of bone marrow graft correlates with time to hematologic reconstitution following autologous bone marrow transplantation with 4-hydroperoxycyclophosphamide-purged bone marrow. Blood. 1997;70:271-275.
18. Brunner AM, Kim HT, Coughlin E, Alyea EP 3rd, Armand P, Ballen KK, Cutler C, Dey BR, Glotzbecker B, Koreth J, McAfee SL, Spitzer TR, Soiffer RJ, Antin JH, Ho VT, Chen YB. Outcomes in Patients Age 70 or Older Undergoing Allogeneic Hematopoietic Stem Cell Transplantation for Hematologic Malignancies. Clinical Research. 2013;19(9):1374-1380.