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PREGLEDNI RAD REVIEW ARTICLE

From nucleated to *ex vivo* manipulated stem cells – an updated biological and clinical synopisis

Od nukleisanih do *ex vivo* manipulisanih matičnih ćelija – osavremenjena biološka i klinička sinapsa

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Abstract

Hematopoietic stem cells (SCs) are responsible for the production and replacement (proliferation) of an extensive quantity of functionally competent blood cells (differentiation) during the entire life, while simultaneously maintaining the ability to reproduce themselves (self-renewal). A complex network of interactive substances and factors organize and protect the survival, maturation and multiplication of SCs.

Hemobiological events in the bone marrow (BM) are synchronized and balanced by the extracellular matrix and microenvironment provided by stromal cells. These cells – including macrophages, fibroblasts, dendritic, endothelial and other cells – stimulate SCs by producing specific hematopoietic growth factors. Other cytokines secreted by stromal cells regulate the adhesion molecules positioned on SCs, allowing them to remain in the BM or migrate to an area where the respective cell type is needed. Thus, hematopoietic SCs could be defined as cells with high proliferative capacity and extensive potential to differentiate into all blood cells or some somatic cell types (SC plasticity) – such as cardiomyocytes, myocytes, osteocytes, chondrocytes, hepatocytes, and even endothelial cells.

Recent increasing clinical use of cell-mediated therapeutic approaches has resulted in increased needs for SCs, but in superior operating procedures during their ex vivo manipulations. The aim of cell harvestings is to obtain a higher SC yield and improved viability or clonogenicity. The goal of optimized cryoinvestigation protocols is to get a minimized cell damages (cryoinjury). Despite the fact that different SC collection protocols and cell freezing practice are already in routine use, a lot of questions related to the optimal SC ex vivo manipulations are still unresolved.

This review summarizes fundamental knowledge and methodological approaches, and recapitulates data enabling progress on constantly evolving research frontiers in the SC area. The studies (including also our investigations) that evaluated the efficiency and safety of SC-treatment (transplants and regenerative medicine) will be also concisely presented.

Key words: stem cells, transplantation, regenerative medicine

Apstrakt

Hematopoetske matične ćelije (MĆ) odgovorne su za produkciju i obnovu (proliferacija) opsežne količine funkcionalno kompetentnih krvnih ćelija (diferencijacija) tokom celog života, istovremeno one održavaju sposobnost sopstvene reprodukcije (samoobnavljanje). Složena mreža interaktivnih supstanci i faktora organizuje i obezbeđuje preživljavanje, sazrevanje i umnožavanje MĆ.

Hemobiološki događaji u kostnoj srži (KS) sinhronizovani/uravnoteženi su ekstracelularnim matriksom i mikrookolinom koji obezbeđuju stromalne ćelije. Ove ćelije – uključujući makrofage, fibroblaste, dendritske, endotelske i druge ćelije – deluju stimulativno na MĆ, oslobađanjem specifičnih hematopoetskih faktora rasta. Neki drugi citokini koje izlučuju stromalne ćelije regulišu adhezivne molekule iskazane na MĆ, omogućavajući im da ostanu u KS ili da migriraju u područje gde je potreban odgovarajući specifičan tip ćelije. Na taj način, hematopoetske MĆ je moguće definisati kao ćelije visokog proliferativnog kapaciteta i ogromnog potencijala da se diferentuju u sve vrste krvnih ćelija ili u neke somatske ćelije (plastičnost MĆ) - kao što su kardiomiociti, miociti, osteociti, hondrociti, hepatociti, ali i endotelske ćelije.

Sve šira klinička primena ćelijski-posredovanih terapijskih pristupa rezultuje stalno rastućim potrebama u samim MĆ, ali i poboljšanjima operativnih procedura njihove ex vivo manipulacije. Osnovni cilj prikupljanja ćelija je da se dobije veći prinos i vijabilnost ili klonogenost MĆ. Cilj optimizovanih protokola krioistraživanja je da se minimizuju oštećenja ćelija (krioоštećenja). Uprkos činjenici da se različiti protokoli za prikupljanje i protokoli kriokonzervacije MĆ su već u rutinskoj upotrebi, brojna pitanja vezana za optimalne ex vivo manipulaciju MĆ još uvek nisu u potpunosti rešena.

Ovaj revijalni rad prikazuje osnovna saznanja i metodološke pristupe, kao i podatke koji omogućavaju napredak na stalno razvijajućim frontovima istraživanja na području MĆ. Studije (uključujući i naša istraživanja) koje procenjuju efikasnost i sigurnost terapijske primene MĆ (transplantacijska i regenerativna medicina) biće takođe ukratko predstavljene.

Ključne reči: matične ćelije, transplantacija, regenerativna medicina

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Stem cells – biology and subtypes

Stem cells (SCs) play essential regenerative roles ranging from embryonic development and organogenesis (embryonic and fetal SCs) to tissue (re)generation (adult SCs). The loss of this well-balanced control sometimes shows tendency towards uncontrolled cell growth or death – thereby developing into a variety of diseases, including tissue defects or cancer (1–3).

The zygote has the maximum peak degree of cell plasticity and it is referred to as a totipotent SC with natural ability of developing into all three types of tissue cells (endoderm, ectoderm and mesoderm). Embryonic SCs are somewhat less plastic and more specialized than zygote. They are also capable of differentiation into all cell types – that is have an option to "switch" into different cell lineages. Thus, inside SC compartment, embryonic SCs are the most "promising", but also the most controversial cell category (2–6).

Immature fetal SCs – as well as the embryonic SCs – can be transplanted into an individual without being rejected. This is because they have little to none of "immune-triggering" proteins – that is HLA antigens on their surface. However, after the 12th gestational week, fetal SCs acquire these proteins, and they remain present on SCs from this point on, including adult SCs (2–4). Consequently, SCs derived from these sources may have therapeutic potential only when given to the individual from whom they were derived/collected (autologous transplants) or from an immunolgically matched donor (allogeneic transplants).

Adult SCs are at a more advanced stage of development. One important point about adult SCs is that there is a very small number of SCs in each tissue. SCs are thought to reside in a specific area of tissues ("niche") where they may remain inactive ("non-dividing") for many years until they are activated by some disease or tissue injury. They can be found in the bone marrow (BM), peripheral blood (PB), blood vessels, fat tissue, skeletal muscles, skin, liver, etc (3–11). Typically, adult SCs are capable of making identical copies (self-renewal) and generate cell types of the tissue in which they reside. However, a number of investigations over the last two decades have raised the possibility that SCs from one tissue may be able to give rise to cell types of some different tissue ("SC-plasticity") (2–6).

Briefly, hematopoiesis is a continuous hemobiological cascade-event (defined also as *in vivo* cell expansion and development) in which from a small amount of SCs a spectrum of committed progenitors/precursors and all mature blood cells are produced and replaced through multi-cyclic processes – such as proliferation or multiplication, and differentiation or maturation, as well as (de)differentiation with (trans) differentiation. These events with a complex network of interactive mediators – grow factors and inhibitors (mainly cytokines) are well regulated (2–4).

Hematopoietic SCs could be characterized as cells having an extensive, but well-balanced self-renewal potential, proliferative and differentiation capacity, as well as ability for cell plasticity. Various populations of SCs expresses CD34 antigen, consequently they are named also as CD34$^+$ cells (2, 5, 11). In the BM about 2–4% of total nucleated cells (TNCs) express the CD34 antigen. The CD34$^+$ cells were detected also in the PB, but in very low ratio in the "steady-state" hematopoiesis: 0.01–0.05% of TNCs (4, 9). In addition, only a minor division of double positive (CD45$^+$/CD34$^+$) cells, with characteristic size and specific intracellular organization – according to the International Society for Hematotherapy and Graft Engineering (ISHAGE – "ish") guidelines for CD34$^+$ cell determination – represents "authentic" SCs (so called SC^{ish}) (4, 9). Immature (more primitive) SC^{ish} expressed CD90 antigen, which is also exposed by 1 – 4% of fetal liver cells, as well as umbilical cord blood (UCB), BM and few PB cells. These cells (CD34$^+$/CD90$^+$ subtype, named as CD90$^+$SC^{ish}) are responsible for stable and long-term BM repopulation (engraftment) with complete hematopoietic reconstitution (2, 5, 9).

Thanks to above mentioned characteristics, hematopoietic SCs provide repopulation of BM after SC transplants. A "traditional" SC transplant involves myelo(immuno)ablative treatment – the use of intensive (radio) chemotherapy as conditioning regimen – followed by (re)infusion of harvested cells in order to eliminate of basic disease, and to get BM repopulation (4–6). Similar procedure with reduced-intensity conditioning (RIC) can be offered to patients disqualified for high-dose chemotherapy because of their age or comorbidity (2, 4).

High-dose chemotherapy followed by allogeneic or autologous SC-transplants is considered as standard treatment for hematologic malignancies (acute lymphoblastic leukemia, acute non-lymphoblastic leukemia, multiple myeloma, Hodgkin's and non-Hodgkin's lymphoma and chronic myeloid leukemia – as an optional therapy), as well as for some non-malignant and immune-mediated diseases (e.g. severe aplastic anemia, multiple sclerosis) – summarized in Table 1 (2–5, 8–12).

The term "regenerative medicine" – created by Haseltine WA in 1999 – is now worldwide used to describe and explain biomedical approaches to heal or restore the body with stimulation of endogenous cells to repair injured tissues, by "implantation" of cells or engineered tissues to replace damaged ones (13, 14). Initial researches and clinical studies in the field of regenerative medicine showed that "implantation" of immature (more primitive) SCs into damaged tissues induces their "homing" and (trans)differentiation into the cell lineages of host organ by "SC-plasticity" (2–5, 15–18).

Some studies have suggested that BM might contain different types of SCs that can produce somatic cells. For example, mesenchymal or stromal cells (MSCs) give rise to osteocytes, chondrocytes, adipocytes and skeletal

Table 1. Current indications and relative suggestions for SC transplant

BM malignant or dysplastic disorders
Leukemias
Hodgkin's and non-Hodgkin's lymphoma
Multiple myeloma
Myelodysplastic/myeloproliferative disorders
Benign immune-mediated disorders
Severe combined immunodeficiency disease (SCID)
Marrow failure syndromes
Severe aplastic anemia
Autoimmune disorders
Solid tumors
Neuroblastoma
Rhabdomyosarcoma
Ewing sarcoma

muscle (3–6). Most recently a novel type of MSCs was isolated from menstrual blood (Menstrual Blood Derived Stem Cells – MenSCs). They have attracted more interest due to their potential therapeutic effects in both experimental models and clinical trials (19).

The idea of "SC-plasticity" have been revised by Ratajczak's group which has recently developed and proved the concept of non-hematopoietic multipotent adult progenitor cells (MAPCs) – such as Very Small Embryonic Like (VSEL) cells (20–23). These cells have practically the same ultrastructural characteristics and protein markers as embryonic SCs. VSEL cells from BM and other organs in non-hematopoietic compartment could be committed to (trans)differentiate into some other tissue, resulting with positive regenerative clinical outcome (22, 23).

Thus, exploring the possibility of using of adult SCs for cell-based therapies in the fields of regenerative medicine has become a very active research area. This is an interesting concept which should be seriously considered in humans.

Stem cells – from harvesting to transplants

In practice, SCs could be collected by multiple aspirations from BM, by mononuclear cell (MNC) harvesting from PB after mobilizing regimen or by purification from UCB. The use of BM or PB derived grafts (allogeneic or autologous) is a standard method in adult transplant setting. UCB transplants have provided hopeful results firstly in pediatric cases – when a matched unrelated BM or PB donor is unavailable. SCs collected from the stated sources can be clinically applied (transplanted) in the treatment of mentioned hematological and/or autoimmune diseases – immediately following harvesting (allogeneic setting) or after a long-term storage in frozen state or cryopreservation (autologous setting) (2, 10, 24–28).

Bone marrow derived stem cells. Historically, BM was the first source for SC transplants (BMT). Cells were collected by multiple aspirations from the iliac crests, under sterile conditions, while the donor was generally anesthetized (Figure 1).

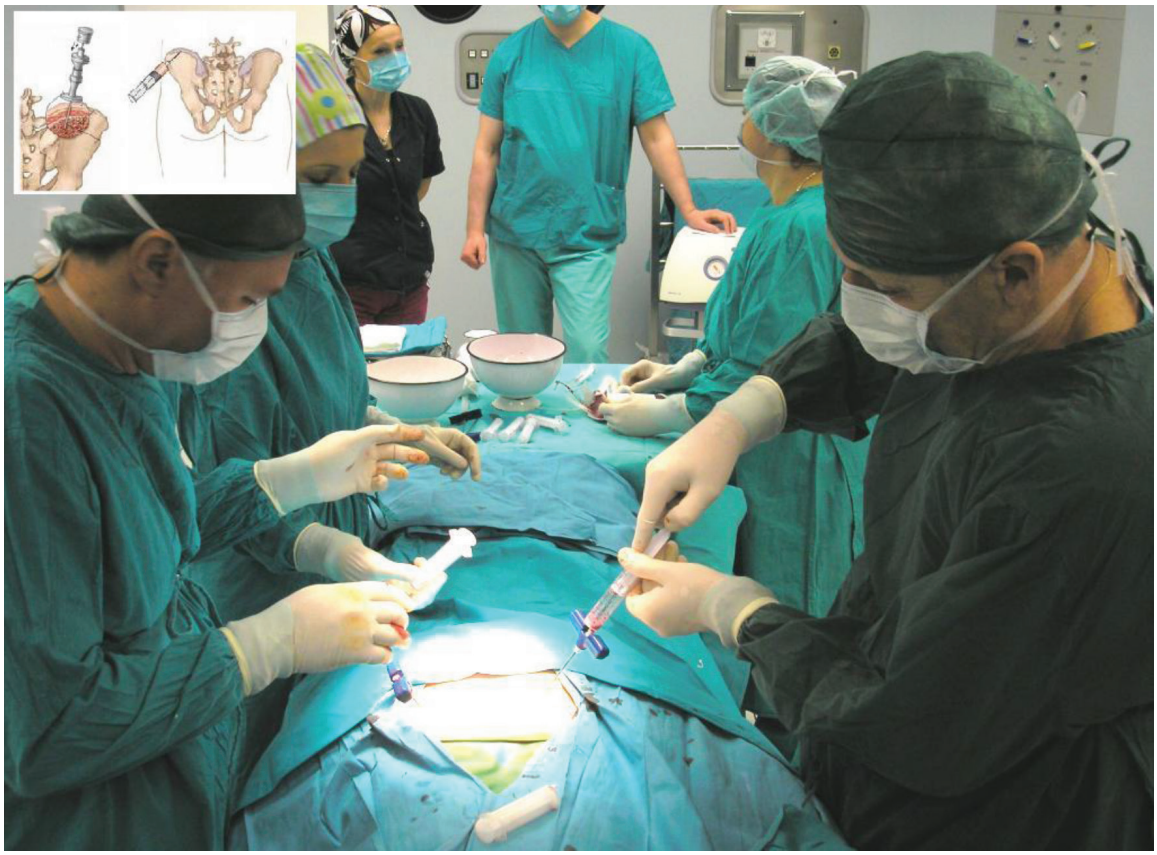

Figure 1. Stem cell collection from G-CSF primed BM by aspirations

The target volume of collected BM aspirate is 10–15 mL per kg of donor body mass (kgbm). In order to provide required number of TNCs (TNC ≥ 3×10^8/kgbm), about 150–200 aspirations are required; a single aspirate volume is 2–5 mL (2). After collection, aspirate should be filtered in order to remove bone and lipid particles and cell aggregates. Anticoagulation is created using citrate solution and heparin (typically 5 000 IU/500 mL) diluted in saline.

Reduction of aspirate volume – precisely, decrease of red blood cell count or plasma quantity – is required for ABO incompatible transplants (processing). Depletion of T-cells in cell suspension is achieved using *ex vivo* purging (cell selection by immunomagnetic technique). These SC purification procedures (processing and purging) enable reduction of red cell for around 80 – 90% and depletion of T-cells with 3–4 Log_{10} (2–4).

In our early BM and PB derived SC research, the ratio of immature ($CD34^+/CD33^-$, $CD34^+/CD38^-$, $CD34^+/DR^-$, $CD34^+/CD90^+$) vs. mature ($CD34^+/CD33^+$, $CD34^+/CD38^+$, $CD34^+/DR^+$, $CD34^+/CD90^-$) $CD34^{+-}$ cell subtypes was compared (2, 10). Data related to cell quantifications are presented in Table 2.

It was found that the collection of superior ratio of immature $CD34^+$ subtypes correlated with complete and long-term BM repopulation (engraftment) and following rapid hematopoietic reconstitution, as well as higher organ repair (regenerative) potential (2, 16).

Peripheral blood derived stem cells. PB derived SC transplant (PBT) could be described by: 1) absence of general anesthesia and less invasive cell collection; 2) low harvest quantity (volume = 200–300 mL) and higher cell yield in the harvest; 3) rapid hematopoietic and immune reconstruction; and 4) inferior transplant-related morbidity. Due to the mentioned reasons, the number of patients treated by PB derived SCs is ever increasing worldwide, especially in autologous transplant setting (5–7, 10).

For harvesting an acceptable SC or $CD34^+$ yield, efficient mobilization protocol is required. Allogeneic donors are given rHuG-CSF 5–10 μg/kgbm daily subcutaneously. The $CD34^+$ cell count in the circulation begins to rise after 3rd day of grow factor injection and peaks is on the 5th day. In autologous setting, patients are given higher rHuG-CSF dose (12–16 μg/kgbm or more daily) combined with chemotherapy (9–11). There are reports of the use of the antagonists for CXC receptor 4 (CXCR4), named as AMD-3100 (Mozobil or Plerixafor) in patients who do not respond adequately to mobilization regimen (4). In our recent study, the use of Plerixafor in combination with rHuG-CSF resulted in more efficient mobilization and following efficacy of cell harvesting as well (11).

In the course of cell harvesting, the determination of the optimal timing of cell collection is a most critical event. For allogeneic transplants, the first SC collection is performed on the 5th day of rHuG-CSF administration. However, an optimized timing for autologous SC harvesting is more complex and controversial. The white blood cell count commonly does not correlate strongly with the $CD34^+$ number in the graft. In contrast, circulating $CD34^+$ count clearly correlates with collection timing and the SC quantity in harvest. It is presented that for a $CD34^+ \geq 20–40/\mu L$ in PB the possibility of the $CD34^+$ yield ≥ 2.5×10^6 cells per kgbm is around 60% or more after one large volume leukapheresis LVL. Of course, higher $CD34^+$ number is found in circulation resulting in superior cell yield (3, 10)

The target $CD34^+$ count should be ≥ $2–4 \times 10^6$/kgbm of the recipient in order to expect successful SC transplant. Recent data support a benefit associated with greater $CD34^+$ yield (≥ 5×10^6/kgbm) compared to the minimum required $CD34^+$ quantity for engraftment (≥ 1×10^6/kgbm) for autologous transplants (3–5).

In our initial PB derived SC investigation the efficacy of LVL and repetitive conventional apheresis (RCA) was evaluated (10). Technical and cellular aspects of aphereses and results obtained by application of different apheretic procedures are presented in Table 3.

Findings obtained in this study suggested that the use of well-timed LVL resulted in superior $CD34^+$ yield, resulting in rapid hematological reconstitution after SC transplants (10). These results required further examinations of the ratio of $CD34^+$ subtypes.

The goal of our most recent SC research was to optimize cell harvesting protocol in order to obtain superior yield of the $CD34^+$ (i.e. SC^{ish}) cells, and especially higher quantity of primitive $CD34^+/CD90^+$ (i.e. $CD90^+SC^{ish}$) cells (9).

As expected, significantly higher ($p<0.001$) absolute count of total SC^{ish} in apheresis product (AP) compared with PB samples was confirmed. Also, the absolute count of $CD90^+SC^{ish}$ was significantly AP ($p<0.05$) higher in AP than in PB, as presented in Figure 2.

Table 2. Distribution of $CD34^+$ subtype markers using double staining

	PB-SCs I	PB-SCs II	BM-SCs
$CD34_{PE}$ / $CD90_{FITC}$ [%]	1.72 ± 1.47	1.25 ± 0.82	2.72 ± 2.06
$CD34_{PE}$ / $CD38_{FITC}$ [%]	2.02 ± 1.18	2.02 ± 1.18	2.30 ± 1.16
$CD34_{PE}$ / HLA-DR_{FITC} [%]	2.01 ± 0.92	2.00 ± 0.92	2.00 ± 0.88
$CD34_{PE}$ / $CD33_{FITC}$ [%]	1.90 ± 1.23	1.90 ± 1.23	2.75 ± 1.12

PB-SCs I = SCs collected from PB mobilized by chemotherapy and rHuG-CSF;
PB-SCs II = SCs collected from PB mobilized using rHuG-CSF alone;
BM-SCs = SCs collected from BM.

Table 3. SC harvesting by apheresis using LVL and RCA procedures

Apheresis type		LVL [n=76]*	RCA [n=20]**
Apheresis procedures	Per whole treatment	1	2.35 ± 0.49
Processed blood volume [L]	Per one treatment	22.45 ± 5.2	9.5 ± 1.4
	Range	12.7–37.8	7.2–12.4
Cell suspension volume [mL]		234.6 ± 55.3	354.6 ± 64.2
$CD34^+$ yield [$\times 10^6$/kgbm]	Per one treatment	12.7 ± 4·8$^{\Psi}$	3.1 ± 1.6$^{\Psi}$
	Total	/	10.8 ± 3.9

*LVL = large volume apheresis;
**RCA = repetitive conventional apheresis;
$^{\Psi}$ $p < 0.05$

In this investigation of relative frequency of $CD90^+SC^{ish}$ demonstrated inverse correlation with the absolute count of total SC^{ish} in both, PB and AP samples. We consider that lower $CD90^+SC^{ish}$ yield in AP is not a consequence of an inferior collection efficacy of our existing apheresis system (Spectra-Optia) for immature $CD90^+SC^{ish}$ cells – as compared to mature SC^{ish} cells from mobilized PB – but most likely result of several still not fully understood/clarified immature SC cytomorphological and/or biophysical features/parameters, such as cytomorphological and biophysical (intracellular granulation, cell-density, etc) (9).

For definitive conclusions further controlled and larger SC-investigations concerning the correlation of circulating and harvested SCs, as well as patients' hematopoietic recovery, are essential.

Umbilical cord blood derived stem cells. UCB is an accepted cell source for pediatric patients and for whom a matched unrelated BM or PB SC donor is unavailable. These "neonatal" SCs are less mature than those in BM. The major advantage of the use of UCB SCs is a non-invasive collection method. Due to the "naive nature" of UCB lymphocytes, UCB grafts do not need to be as "rigorously" matched to a recipient as BM or PB grafts. The disadvantage of this cell source is the limited SCs count (around 3×10^6 $CD34^+$ per unit) (2–6, 22, 24). Since SCs in the UCB are "more primitive", the engraftment process takes longer with UCB – leaving the patient vulnerable to posttransplant infections or bleeding. However, "more primitive" SCs in UCB have the potential to give rise to non-hematopoietic cells (myocardial, neural and endothelial cells, etc) by (trans)differentiation (3, 22).

The aim of our study was to compare two different UCB collection techniques (first one with the original Syringe/Flush/Syringe system and the second one using the standard method by gravity) and to evaluate UCB derived SC cryopreservation protocols (29). These results documented that it is possible to improve *in utero* collection strategy using Syringe/Flush/Syringe system before placental delivery – greater volumes of UCB units compared to standard gravity technique. Findings also confirmed the best recovery of UCB cells when controlled-rate freezing procedure and 5% dimethyl sulfoxide (DMSO) was combined (29).

Stem cell cryopreservation. The successful SC transplant requires both efficient collection by aspirations or apheresis and cryopreservation techniques for obtaining an optimized SC yield and recovery – with minimized cell injuries during the freeze/thaw process (cryoinjury) (2–4, 30).

Microprocessor-restricted (controlled-rate) freezing is a time-consuming process, based upon high-level technical expertise. Uncontrolled-rate ("dump-freeze" without programmed cooling rate) technique is less costly because it does not require a programmed

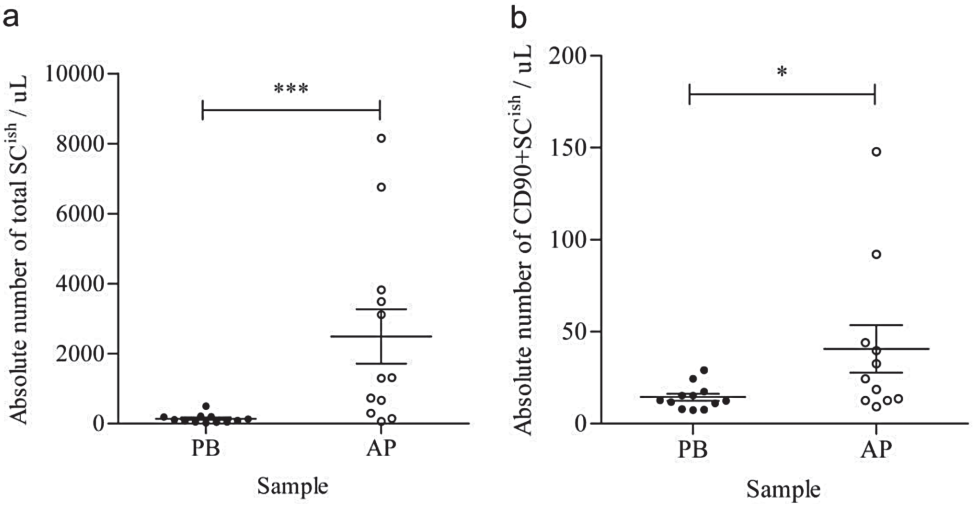

Figure 2. The ratio of the SC^{ish} and $CD90^+SC^{ish}$ cells in PB and harvest (apheresis product)

freezing-device. However, there are indications that controlled-rate method is a superior alternative to uncontrolled-rate technique due to higher quantitative and functional recovery of cells (2, 10, 16). For obtaining an effective cryopreservation, besides specific, i.e. optimized freezing method, the choice and use of appropriate cryoprotectant agent is required. At present, for SC and platelet freezing, DMSO and HES are commonly used as cryoprotectants, although in different concentrations (2, 4).

Our early cryoinvestigation (carried out on CBA/H-mice model) demonstrated that recovery of very primitive pluripotent SCs (Marrow Repopulating Ability – MRA) is the highest when DMSO in higher concentration (10%) was applied. These results imply a different "cryobiological request" of MRA cells in comparison with the committed progenitors (CFU–S and CFU–GM) (30).

As we earlier presented, "conventional" SC transplants were used in the treatment of patients with acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), imatinib-refractory chronic myelogenous leukemia (CML), multiple myeloma (MM), Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL) and some benign blood diseases, as well as some autoimmune disorders (2, 10–12, 25–28).

In our earlier studies (2, 8, 10), different SC sources (PB vs. BM) and two harvesting techniques (LVL vs. RCA) – on the basis of $CD34^+$ cell yields and various clinical data – were evaluated. The majority of patients (76.7%) had infused with more than $5.0x10^6$/kgbm $CD34^+$ cells, while 68.3% of patients treated by $4.0–5.4 \times 10^6$/kgbm $CD34^+$ dose, respectively (11). The speed of hematopoietic reconstitution was presented in Figure 3.

Hematopoietic reconstitution was obtained earlier, that is on the 11.4^{th} vs. 15.9^{th} day (for granulocytes) and the 14.1^{th} vs. 17.5^{th} day (for platelets) when PBT vs. BMT were compared. As well, faster hematopoietic reconstitution was registered (9.4^{th} and 12.4^{th} day for leukocytes and platelets, respectively) when LVL harvesting was used. However, there were no clinically relevant intergroup (LVL vs. CRA) differences in these patients (2, 10).

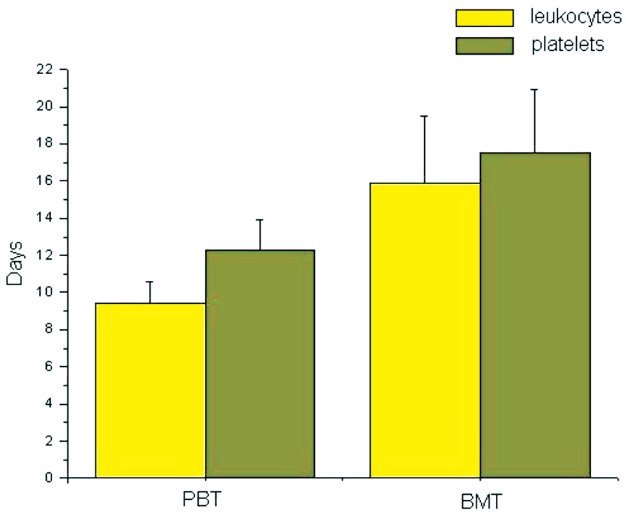

Figure 3. The rapidity of hematopoietic reconstitution following PBT vs. BMT

Finally, the results from these studies undoubtedly confirmed that applied $CD34^+$ cell dose is an independent factor that may contribute to superior clinical outcome and overall survival of patients following transplants (11).

Stem cells for cardiac repair

The concept of SCs plasticity enables their rising therapeutic use in regenerative medicine (ischemic heart disease, liver damage, osteogenesis imperfecta, etc). Different mechanisms that enable SCs (trans) differentiation and "cell-reprogramming" – regulated and/or mediated by "extrinsic" or "intrinsic" factors – are only partially explained (31–36).

The left ventricle dilatation occurs in even approximately one third of the patients reperfused effectively with primary angioplasty. Thus, it is imperative to develop a therapeutic approach to prevent of myocardium remodeling. The SC therapy is a new and promising method of an infarcted heart healing (36–38).

The primary natural or (patho)physiological source of autologous "regenerative cells" is perhaps the cardiac SC compartment which are in inactive ("non-dividing") phase – but following acute myocardial infarction (AMI) could be differentiate into cardiomyocytes and endothelial cells. Myocardial ischemia after AMI initiates release of various cytokines and chemokines – which induce cell mobilization from other SC "niches" and their homing into the damaged myocardium. In clinical practice, BM is the most frequent source of cells used for cardiac repair (5, 36).

Our initial clinical study from 2004 (37), contains patients with a major first anterior wall infarction with a LVEF < 40% on the 5^{th} day. The percutaneous intracoronary injection of BM derived MNCs (with presence of SCs – MNC/SCs) into the left coronary artery was performed in the second week after AMI. Patients who had a large infarct zone with a large increase in lactate dehydrogenase had no significant reduction in the infarct zone, nor a significant LVEF recovery after 6 months. However, it has been found that about 50% of patients can expect an improvement of the LVEF by ≥ 5% and a reduction in the infarct zone by ≥ 5% over a 6 month period (37).

In our subsequent study (38) the LVEF was improved after 4 months in group of patients treated with MNC/SCs and in control group (without cell-therapy), but did not reach statistical significance in the group treated with G-CSF primed BM derived SCs because of small number of patients in that group (n=5). The infarction zone size has the same pattern. Difference between baseline and 6 months infarction zone size was significant ($p<0.01$) in patients with

Table 4. Infarction size end left ventricle ejection fraction baseline vs. after 4 months

Parameters	MNC/SC therapy of AMI (n=19)	G-CSF primed BM derived SCs group (n=5)	Control group – without cell-therapy (n=17)
Infarction size at baseline (LV%±SD)	28.4±11.3	35.6±8.0*	31.4±12.8
Infarction size +6 months (LV%±SD)	25.2±12.6	25.2±8.6*	27.9±10.7
LVEF at baseline (% ± SD)	32.9±4.1	36.4±3.0	34.3±5.2
LVEF +4 months (% ± SD)	37.0±9.0	43.8±3.0	36.9±8.2

LV= left ventricle; LVEF = left ventricle ejection fraction; *p<0.01.

G-CSF primed BM derived SC group alone. There was no significant difference between the change of LVEF at baseline and after 4 months (Table 4).

Our preliminary results have shown that G-CSF primed BM derived SC treatment was safe with two-three times higher number of MNCs applied and there was a trend toward larger increase of 4-months ejection fraction and greater decrease of the infarction size than the control groups. Any procedure that increases the left ventricle ejection fraction for > 5% after a several months follow-up could be of absolute clinical and economic importance/advantage. However, we need a lot of basic research and randomized clinical trials to define the exact role of G-CSF primed BM derived SC treatment for ischemic heart disease (5, 36–38).

The use of MNC/SCs – implanted into myocardium during coronary artery bypass grafting (CABG) for treatment of ischemic cardiomyopathy patients planned for CABG surgery in our Center in 2006 was started.

The object of our recent study (39) was to test the hypothesis that intramyocardial MNC/SC implantation associated with CABG surgery leads to better postoperative results than CABG surgery alone – regarding the "primary-end-point": patients functional capacity and the "secondary-end-point": cardiovascular mortality in the follow-up period of 5 years. Six minute walk test (6-MWT) was used to evaluate patient's functional capacity. The 6-MWT was not performed preoperatively due to poor condition, dyspnea and/or angina at rest.

The value of this clinical study was confirmed also by Ayyat's meta-analysis (40) – in which our investigation was accepted and positively presented (Figure 4).

Summary of included trials characteristics

Trial (year)	Country	Sample n, BMSC/ Control	Age (mean) years		Male n		Number of Cells (mean ± SD)	Route of Injection	Follow-up months	Imaging Modality
			BMSC	Control	BMSC	Control				
Ang et al. 2008 (37)	UK	21/21/20*	64.7/62.1	61.3	15/19	18	84 ± 56 × 10^6/115 ± 73 × 10^6 BMSC	IM/IC	6	MRI
Hendrikx et al. 2006 (40)	Belgium	10/10	63.2	66.8	10	7	60.25 ± 31.35 × 10^6 BMSC	IM	4	MRI
Hu et al. 2011 (18)	China	31/29	56.6	58.2	29	27	131.7 ± 106.6 × 10^6 BMSC	IC	6	MRI
Maureira et al. 2012 (20)	France	7/7	58.0	57.0	7	6	342 ± 42 × 10^6 BMSC	IM	6	MRI
Naseri et al. 2018 (14)	Iran	30/21/26§	53.1/51.5	55.5	19/27	23	564 ± 69.4 × 10^6 MNC / 8.2 ± 4.3 × 10^6 $CD133^+$	IM	6	SPECT
Nasseri et al. 2014 (38)	Germany	30/30	62.7	62.7	28	29	6.1 ± 4.52 × 10^6 $CD133^+$ BMSC	IM	6	MRI
Noiseux et al. 2016 (13)	Canada	19/14	66.4	63.1	17	13	6.5 ± 3.1 × 10^6 $CD133^+$ BMSC	IM	6	MRI
Patel et al. 2005 (39)	Argentina	10/10	64.8	63.6	8	8	22 × 10^6 $CD34^+$ BMSC ‡	IM	6	Echo
Steinhoff et al. 2017 (12)	Germany	28/30	64.0	63.6	26	26	2.29 ± 1.42 × 10^6 $CD133^+$ BMSC	IM	6	MRI
Trifunovic et al. 2015 (17)	Serbia	15/15	53.8	60.0	14	14	2.65 ± 1.71 × 10^6 BMSC	IM	6	Echo
Wang et al. 2015 (41)	China	45/45	61.4	62.9	37	35	521 ± 44 × 10^6 BMSC	IM	6	Echo
Wang et al. 2016 (42)	China	17/16	65.6	65.5	8	7	98.5 ± 48.3 × 10^6 BMSC	IM	6	Echo
Zhao et al. 2008 (43)	China	18/18	60.3	59.1	15	15	659 ± 512 × 10^6 BMSC	IM	6	Echo

BMSC, bone marrow stem cells; IM, intra-myocardial; IC, intra-coronary; MNC, mononuclear cells; MRI, magnetic resonance imaging; Echo, echocardiography.

Figure 4. Data from meta-analysis of the MNC/SC application in cardiosurgery by Ayyat's group

We documented that the use of MNC/SCs with CABG is a safe and reasonable therapeutic method that demonstrated not only the improved functional capacity and recovered LVEF, but also a reduced long-term cardiac mortality of patients in follow-up period (39).

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The intensification of SC transplants and other cell-mediated approaches have resulted in elevated needs for higher quantity of SCs and improved operating procedures to minimize cell damages. Thus, the potential of long-term SC engrafting/autografting in future will depend on the development of optimized harvesting protocols, extracorporeal "graft-engineering" and advanced anti-cancer approaches. The SC (trans)differentiation could lead to their extensive use in regenerative medicine.

As well, SCs are considered as potential targets for gene therapy or gene transduction due to their ability to renew themselves – i.e. generate a "self-perpetuating" cell population that contains the transduced gene for the patient's lifetime. Diseases that could be candidates for gene therapy include thalassemia, sickle cell anemia, SCID, Gaucher's disease and a variety of metabolic deficiencies – but in this field of medicine even now the number of potential questions is higher than the number of possible answers.

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