STEM CELL TRANSPLANT: FROM CELL HARVESTING TO CRYOPRESERVATION

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Summary

Stem cells could be defined as cells capable for self-renewal with high proliferative capacity and extensive potential to differentiate into blood cells or some somatic cell types – “plasticity” due to “trans-differentiation” such as osteocytes, chondrocytes, hepatocytes, myocytes, cardiomyocytes and even endothelial cells. Recent increasing clinical use of various cell-mediated therapeutic approaches has resulted in amplified needs for both stem cells and operating procedures to get a minimized cell damages during collection, purification and cryopreservation. The aim of cell harvesting procedures is to obtain the best stem cells yield, high purity and good viability/colonogenicity. The goal of optimized cryoinvestigations protocols is to minimize cell injuries during the freeze/thaw process (cryoinjury). Despite the fact that different stem cells collection protocols and cell freezing practice are already in routine use, a lot of questions related to the optimal blood stem cells harvesting, purification and cryopreservation are still unresolved.

Key words: Hematopoietic Stem Cell Transplantation; Cell Culture Techniques; Stem Cells; Cryopreservation; Cell Separation; Transplantation, Autologous; Dimethyl Sulfoxide; Blood Preservation

Introduction

Hematopoiesis is a continuing/steady event through which many different blood cells are produced from a small number of stem cells (SCs) by proliferation and differentiation. A multifactorious network of interactive mediators controls the survival (self-renewal), proliferation and differentiation of SCs in bone marrow (BM), including extracellular matrix and microenvironment provided by stromal cells [1, 2]. These cells – macrophages, fibroblasts, dendritic, endothelial and other cells – stimulate SCs by producing hematopoietic cytokines, such as SC-factor, interleukins, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and other growth factors [1–3]. Immature SCs have a capacity for self-renewal, a high potential for proliferation and differentiation into pluripotent or committed progenitors and mature blood cells. Thanks to described nature, SCs provide complete and durable or late BM repopulation and hematopoietic reconstitution following transplants. The SC transplants involve myeloablation (high-dose chemotherapy), followed by (re)infusion of harvested autologous or allogeneic cells. Similar procedure with reduced-intensity conditioning (RIC) can be offered to patients who are disqualified for intensive conditioning protocol – because of their age or comorbidity [1–4].

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Abbreviations

ISHAGE – International Society for Hematotherapy and Graft Engineering
SC – stem cell
MSC – mesenchymal stem cell
GM-CSF – granulocyte-macrophage colony-stimulating factor
M-CSF – granulocyte colony-stimulating factor
DMSO – dimethyl sulfoxide
HES – hydroxyethyl starch
ACD – acid-citrate-dextrose
TNC – total nucleated cell
MNC – mononuclear cell
LVL – large volume leukapheresis
GvHD – graft versus host disease
CFU-S – colony-forming unit spleen
CFU-GM – colony-forming unit granulocyte-macrophage

Generally, SCs can be divided into embryonic and adult cell compartment, but several adult SCs have a similar high-level ("unlimited") biological potential to embryonic cells [2, 4]. Thus, some adult SCs are able to develop into a variety of somatic cells by using the features of "plasticity" or "trans-differentiation" [5–9]. Although the term "plasticity" has become very popular, some studies have suggested that BM might contain different types of SCs that can produce non-hematopoietic (somatic) cells. For example, mesenchymal SCs (MSCs)/pericytes (that is contractile cells that wrap around endothelial cells) from BM can switch to osteocytes, chondrocytes, adipocytes and skeletal muscle cells [2, 4, 8]. Consequently, these cells are applicable in the field of regenerative medicine, i.e. organ repair/regeneration.

In practice, allogeneic transplants require both an effective conditioning regimen, as well as optimized mobilization and harvesting protocol to obtain adequate SC yield [10–14]. For autologous transplants, the use of an optimized cryopreservation procedure guarantees the best recovery of thawed cell [15–21]. Despite the fact that cryopreservation is already in routine use, some questions related to the best freezing method and cryoprotective agents (dimethyl sulfoxide – DMSO and hydroxyethyl starch – HES) type and their optimized concentration are not precisely answered [2, 4, 22].

The intensifying of myelo- (immune-) ablative therapy combined with SC transplant and the introduction of cell-mediated restorative/regenerative methods (“cell-therapy”) resulted in increased needs for both SCs conceptual and practical operating procedures inducing minimized cell damages during their harvesting and cryopreservation. In this article, we will briefly review the practical aspects of an optimized harvesting protocol, processing and/or immunomagnetic selection, as well as cryopreservation of the SCs. Our results of the SC investigations will also be summarized.

Stem cell sources and harvesting

Historically, BM was the first source of SCs for transplant in experimental and clinical setting [4, 23–25]. The BM aspirate collection is the same for an allogeneic donor as for an autologous (occasionally) patient. SCs are harvested by multiple aspirations from the posterior and anterior iliac crest (Figure 1).

The procedure is performed under sterile conditions, while the donor is generally anesthetized. Immediately after collection, aspirate should be filtered in order to remove bone and lipid particles and/or cell aggregates. Anticoagulation is provided by acid-citrate-dextrose formula B (ACD-B; 1.8% citrate concentration) and by heparin diluted in saline (5000 IU/500 mL) [2, 4].

In order to obtain a required total nucleated cell (TNC) yield – that is 3 × 10^8/kg of body mass (kgbm) of recipient – the target/maximal volume of collected aspirate is 10–15 mL per kg bm of donor (total volume = 800–1000 mL). For ABO incompatible (major and/or minor) transplants, red blood cells number (RBC) and/or plasma quantity reduction is required. Cell purification procedures such as processing and selection, enable reduction of the aspirate volume and decrease of RBC quantity (depletion = 80–90%; processing), as well as CD34+ cell enrichment (positive selection) or T-cell depletion (efficacy 3–4 Log10; negative selection) [4, 16, 26]. In the aspirate, approximately 2–4% of TNCs express the CD34 antigen [14]. Shortly, CD34 is the cluster designation given to a transmembrane glycoprotein present on SC surface and some stromal cells. Cells expressing the stated CD34 and CD90 antigens are capable of complete and durable or late reconstitution of hematopoiesis [2, 4].

The collection of SCs from PB (PB-SCs) is an aphaeretic procedure with respect to the standardized protocol and cell yield. The CD34+ cells are documented in peripheral blood (PB) in the “steady-state” hematopoiesis also (in very low percentage: 0.01–0.05% per TNCs) [4, 14]. The first collections of “steady state” PB-SCs were performed using 6 to 9 collections and additional cryopreservation was required [2].

Mobilization by chemotherapy and recombinant G-CSF extensively increases the quantity of circulat-

Figure 1. Stem cell collection from bone marrow by multiple aspirations
Slika 1. Prikupljanje matičnih čelija multiplim aspiracijama
ing CD34+ cell count in patients or donors. However, just a small fraction of CD45+/CD34+ cells, with typical size and specific intracellular granularity – according to the ISHAGE protocol are “authentic” SCs [14]. The immature CD34+ cells (CD34+/CD90− subset) infused can more precisely predict leukocyte and/or platelet (Plt) recovery after SC-transplant. In practice, SC-engraftment is defined as neutrophil number ≥ 0.5 x 10⁹/L and Plt count exceeding 20 x 10⁹/L (without blood component support) [2, 4, 14].

The main benefits of PB-SC harvestings/transplants are the absence of general anesthesia, higher CD34+ yield, rapid hematopoietic reconstitution and smaller transplant-related morbidity. As a result, the number of patients transplanted by PB-SCs is ever increasing worldwide, especially in autologous SC transplant setting (about 80% of allogeneic and almost of 100% autologous transplants). On the other hand, the main disadvantage of the use of PB-SCs is high-level T-cell quantity with following elevated risk of graft versus host disease (GvHD), as well as possible “contamination” of harvest with tumor cell [2–4].

Nowadays, the typical number of apheresis required is not more than one to three, and for anticoagulation ACD–B or ACD–A (with 2.2% citrate concentration) are used [10–14] (Figure 2).

For allogeneic transplants, vascular access is typically realized through ante-cubical veins. In autologous setting, collection should be performed by the central-venous vascular access across catheters. The use of catheters simplifies harvesting, but may be associated with topical thrombosis. Finally, there is approximately one percent central-venous catheter-related hazard of the local infection, pneumothorax or bleeding [2, 4].

As mentioned, for obtaining adequate SC or CD34+ yield, efficient mobilization protocol is required. Allogeneic donors are given G-CSF 5–10 μg/kgbm daily subcutaneously. The CD34+ cell count in the circulation begins to rise after 2–3 days of G-CSF administration and peaks on the fifth day [2, 4]. When donor mobilization with G-CSF is poor, the only way to improve yields is to increase the blood volume processed or the number of collections. In autologous donors, doses of recombinant growth factor are higher – patients are given 12–16 μg/kgbm G-CSF daily, combined with chemotherapy (cyclophosphamide 4–7 g/m²) or by poly-chemotherapy in corresponding doses [2–4].

The determination of optimized collection timing is very important and a critical event for PB-SC harvesting. Usually, in allogeneic setting the first (or single) harvesting is on the fifth day of G-CSF administration [2, 4]. The definition of optimized timing for autologous collection is more complex and controversial. The optimal harvesting timing can be determined based on the leukocyte, mononuclear cell (MNC) counts, as well as the number of circulating CD34+ cells. The optimal time to begin cell collection in is when the leukocyte count is between 5–10 x 10⁹/L. However, the leukocytes do not correlate strongly with the number of SC or CD34+ yield in the harvest. Opposite, CD34+ cell count in PB clearly correlates with harvesting timing and the SC or CD34+ quantity in the harvest (as a function of the volume of blood processed also). Precisely, it was demonstrated that for a CD34+ ≥ 20–40 μL of patient's PB the possibility of the CD34+ yield in harvest ≥ 2.5 x 10⁹ cells/kgbm is about 60% or more using one large volume leukapheresis (LVL). Of course, higher CD34+ number in circulation results in superior yield [2–4, 12–14].

Some patients who have previously been treated with high-dose chemotherapy may be “poor responders” for mobilization. The most efficient approach to obtain adequate SCs from “poor-mobilizers” is not resolve still. Simultaneous collection of SCs from BM and PB has not improved the engraftment rate considerably. Increased doses of G-CSF or use of G-CSF together with GM-CSF has also effectively mobilized some autologous donors. Finally, there are data describing the superior effects of some new agents in combination with G-CSF, such as plerixafor or mozobil (antagonist of the alpha chemokine receptor CXCR4) in mobilizing the CD34+ cells – including the immature SCs, capable for durable or late (long-term) BM repopulation with following hematopoietic reconstitution [2, 14].

Our results confirmed high-level efficacy of the LVL. Namely, for the 89.5% patients using one LVL, the mean CD34+ yield was 12.1 x 10⁹/kgbm (allogeneic) and 6.5 x 10⁹/kgbm (autologous), respectively. In our group of patients, the circulating CD34+ count was also relatively high 40–60 μL following mobilizing regimen [3, 10–14]. Our investigations also verified that CD34+ post-selection cell recovery was 70–80%, when CD34+ purity (CD34+ cell percentage in final cell suspension) was around 80–90% [2–4]. In addition, our latest research demonstrated inverse correlation of the CD34+/CD90− frequency with the absolute count of total CD34+ cells in PB and the harvest. We considered that poorer CD34+/CD90− yield in the harvest is not a outcome of an inferior harvesting efficacy – but most likely result of several even now not fully resolved immature SC cytomorphological and biophysical features [14].

Figure 2. Stem cell harvesting using blood cell separators
Slika 2. Prikupljanje matičnih čelija primenom separatoa krvnih čelija
Cryopreservation of stem cells

The use of cryobiology for cell preservation began in 1949 with the freezing of sperm cells, using glycerol as a cryoprotective agent [15]. Afterwards, DMSO and HES techniques were applied for cryopreservation of different blood-derived nucleated cells and/or Plts [16, 18, 19, 22].

Controlled-rate or microprocessor-restricted freezing is a time-consuming process, which requires high-level technical expertise. Uncontrolled-rate or “dump-freeze” technique (without programmed cooling rate) is less expensive because it does not require a programmed freezing-device. However, the controlled-rate method is an advanced alternative to the uncontrolled-rate system due to superior quantitative, morphological, ultrastructural and functional cell recovery [17–21].

The basic goal of cryoinvestigations was to predict the cell response to freeze/thaw processes and cryoprotective agent addition/removal, as well as evaluation of cryobiological variables such as biophysical, physicochemical and other parameters responsible for cryoinjury of living cells. As stated, SC cryopreservation is nowadays in routine practice, but recent cryoinvestigations suggest that freezing strategies should be revised to optimize specific cryobiological systems to minimize the cryoinjuries and maximize cell recovery. Cryoinjuries can be detected as cell lesions, caused by the decrease of selected functions to the total cell destruction (cytolysis). At present, cryoinjuries are considered to result from the extensive volume reduction (cellular dehydration or solution effect) and/or massive intracellular ice crystallization (mechanical damage). These mechanisms can act together — the first event is expressed primarily at low-rate (≤ 10 °C/min) freezing, and the second one at high-rate (≥ 10 °C/min) freezing [2, 4, 17].

Thus, to establish an optimized cooling rate during cell freezing, specific for each cell type and cryobiological system should be considered. The speed of cooling should be high enough to prevent cell dehydration and adequately low to enable the efflux of water from the cell. It would be ideal to find a cooling rate just less than the one, which causes intracellular crystallization [4, 17]. An optimal freezing rate is the function of the ratio between cell surface versus volume and cellular membrane permeability for water and its corresponding temperature coefficient — but it also depends on what type of cryopreservation strategy is applied. Last but not least, a higher degree of cell destruction has occurred when transition period from liquid to solid phase (fusion heat releasing) is prolonged. The released heat of fusion — if not considered during controlled-rate freezing — could result in additional temperature fluctuation. That is why the period of transformation from liquid to solid phase will be prolonged, and its duration is directly related to the degree of cryoinjury [2, 4, 18–21].

An optimized (controlled-rate vs. uncontrolled-rate) cell freezing approach cannot solve all problems related to cryoinjuries — post-thaw cell recovery and viability are high only when cryoprotective agents are present in the cryobiological system. They prevent or reduce the degree of cell thermal damages. In brief, cryoprotective agents can demonstrate protective effects by the reduction of cell dehydration, as well as by decreasing the intensity of intracellular crystallization. Cryoprotective agents are classified into intracellular (penetrating) and extracellular (non-penetrating) compounds. Mechanisms of their action are complex and only partially recognized. Due to the differences in its chemical and other properties, it is not possible to determine a cryoprotective mechanism common for all cryoprotective agents. In brief, extracellular agents could protect cells during rapid freezing, reducing the intracellular ice crystal formation. Opposite, intracellular cryoprotective agents could provide protection in the course of low-rate freezing, decreasing the degree of cell dehydration controlled-rate freezing [2–4, 17, 18, 21].

In practice, PB-SC cryopreservation consists of the following steps: 1) equilibration (cell exposure to cryoprotectant — DMSO); 2) freezing process (controlled-rate or uncontrolled-rate system); 3) cell storage at -90±5 °C (mechanical freezer) or at temperature from -120 °C to -150 °C (mechanical freezer/steam of nitrogen) or at -196 °C (liquid nitrogen); and 4) cell thawing in a water bath at 37±3 °C (Figure 3) [2–4].

Our earlier cryoinvestigations demonstrated that the recovery of pluripotent and committed hematopoietic progenitors (CFU-Sd12 and CFU-GM) in the presence of lower concentration of cryoprotective agent (5% vs. 10% DMSO) was superior. However, it has also been confirmed that the recovery of very primitive pluripotent (Marrow Repopulating Ability — MRA) hematopoietic SCs was better when 10% DMSO used. These results imply a different...
Hodgkin’s lymphoma resulted with rapid hematopoietic reconstitution [2–4, 8, 12].

Conclusion

Despite the fact that different stem cells collection protocols and cell freezing practice are already in routine use, a lot of questions related to the optimal blood stem cells harvesting, purification and cryopreservation are still unresolved.

References