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Cellular cryobiology – a review of basic concepts and "operating design" of cryopreserved cells

Celularna kriobiologija – prikaz osnovnih koncepata i „operativnog dizajna" kriokonzervisanih ćelija

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Introduction

Cell preservation systems could be classified into the following three categories: 1) liquid-state conservation (at hypothermic condition, but over 0 °C; 2) frozen-state storage at ultra-low temperatures (cryostorage); 3) cell cultivation in an artificial medium (normothermic storage). Cell or tissue cultures need nutrients, buffers, and other supplements for metabolism to preserve cells in a "near-normal" (comparatively physiological) condition. On the contrary, the purpose of cell cooling (refrigeration) is to reduce cell needs for energy production and its consumption (for protein synthesis, ion transport, and other biochemical activities) at long-term storage [1].

Cryobiology is an innovative scientific discipline that evaluates influences of subzero (≤ 0 C°) and ultra-low (-80 ± 5 °C or lower) temperatures on cell integrity and functionality, as well as determines facts/approaches applicable in cryo-practice. Cryopreservation (CP) is a thermodynamically well-defined operating system, specific for each cell type or "biosubstratum", that protects cellular organelles, isolated cells, cell clusters, or tissues during cooling to ultra-low temperatures. This method is beneficial when cells are biologically and/or thermally unstable using liquid-state cell preservation [2–4].

The key CP requests are to minimize cellular thermal damages (cryoinjury-score) and their consequences after the freezing/thawing procedure for each "cryo-biosystem" (frozen biological systems). Briefly, it is a specific system composed of some cells ("biosubstratum"), medium, and cryoprotective agent (CA) [cryoprotectant (CPt)] [2–5]. Different CP systems have been described over the past decades: 1) ultra-rapid cooling/thawing technique (with no obligatory use of a CPt); 2) freezing by nonpenetrating polymers [cooling rate (CR)] could be slower and relatively uncontrolled; 3) vitrification method (for CP of tissues when even extracellular freezing process induces unacceptable or intolerable cryoinjuries); 4) equilibrium-freezing (the "cryo-biosystem" must contain enough CA to reduce extracellular freezing and avoid osmotic stress, as well as intracellular ice formation until vitrification is developing) [4–11].

This paper is a review of basic events of physicochemical/biophysical phenomena within workable protocols used in both theoretical and practical cell CP approaches. There

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fore, it could be a synopsis of factors relevant for affecting the quantitative/qualitative recovery of various cells exposed to ultra-low temperatures. The efficacy of our original CP protocol, based on research data and clinical evaluation of the cells applied, will also be briefly described.

Initial cellular cryo-investigations

The ever-increasing use of cell-mediated treatments has resulted in increased needs for both stem cells (SCs) and other immunoreactive cells, but also for superior *ex-vivo* manipulative/operating procedures to minimize cell damage during their collection, processing, and storage in liquid or frozen state. The objective of cryo-investigations is to determine critical events in cryo-practice, predict cell response to the cooling/thawing process, and CPt addition/removal, maximizing post-thaw cell quantity/quality [3–5].

The epoch of cryobiology began in 1949 by CP of the fowl spermatozoa using glycerol as a low molecular weight (MW) CPt [6]. Then, methods using glycerol and dimethyl sulfoxide (DMSO) were initiated for freezing human hematopoietic progenitors and blood cells [7–10]. In brief, glycerol is a potent stabilizer of different macromolecules, and it is non-toxic to the majority of cells, even at high-level concentrations. The major inconvenience of this CPt is that glycerol diffuses (penetrates) into a lot of cells just gradually and/or relatively in slow motion. DMSO has the benefit of more rapid (prompt) penetration into the majority of cells, but it is toxic in higher concentrations [4, 5].

Today, several CP protocols are in clinical use, but a universal optimized freezing procedure based on adequate type and concentration of CPt has not yet been achieved. Freezing/thawing systems applicable in medical cryo-practice should be persistently improved to minimize thermal damages and maximize cell recovery and viability. In this context, re-evaluation of biophysical and biochemical factors (including osmotic characteristics, water/CPt permeability coefficients), as well as other cryobiological parameters responsible for cryoinjuries, is still a very popular topic for investigation by researchers and practitioners [5, 11–16].

Cell cryoinjury – initiation and manifestations

Thermal injury can be manifested as partial cellular lesions due to various malfunction(s) or as complete cell destruction or cytolysis. Generally, post-thaw cell recovery/viability is better when correct freezing methods and high-quality CPt are applied. The essential factors that can result in superior cryoprotection during living cell CP are the following: 1) the use of an effective freezing system, such as optimized controlled-rate freezing with compensation of the released fusion heat during the "phase transition" step (from liquid to solid stage); 2) the determination of acceptable cryostorage category and conditions (adequate temperature and length of cryostorage); 3) the selection of practical thawing technique; 4) the choice of an appropriate type and concentration of CA. For SCs, progenitor and mature blood cell CP, glycerol, DMSO, or hydroxyethylstarch (HES) – a high-molecular-mass agent, are regularly used, although in different combinations and concentrations [5, 14–17].

Initially, it was believed that cryoinjuries emerge predominantly because of the consequent occurrence of extracellular ice crystals. It was supposed that the application of a sufficiently high cooling speed (CS) might avoid extracellular ice formation and later cell destruction. However, an extremely high CS (adequate to prevent cell damage) could not be realized in practice because of the heat transfer thermodynamic limitations. Additionally, complete cell destruction was observed using ultra-rapid freezing procedures [2–5].

At present, it is considered that cell thermal damage during the freeze-thaw process may be the result of high-level cell dehydration with subsequent high-level volume reduction ("solution effect") or development of extensive intracellular ice formation followed by resulting organelle and membrane damages ("mechanical damage"). The first mechanism is typically expressed during low-rate freezing as a result of a progressive rise in the osmotic gradient between extracellular and intracellular space. Extracellular hypertonicity is followed by subsequent cell dehydration "triggering" volume decrease, membrane malformations, and, lastly, cytolysis. Extracellular ice crystals do not regularly induce mechanical cell destruction due to membrane penetration despite their physical presence. The second process is characteristic of rapid or high-rate freezing when the intracellular water cannot leak out of the cell (absence of fluid efflux). Intracellular ice formation, followed by cell destruction, is the most critical harmful effect. These phenomena ("solution effect" and "mechanical damage") are independent cryo-events but can sometimes affect and work together and typically result in cell destruction [5, 14–16].

Recent cryo-investigations (including ours) are primarily focused on the evaluation of controlled-rate freezing (microprocessor restricted/programmed cooling) vs. uncontrolled-rate freezing ("dump-freezing" without programmed CR) techniques; for that reason, only those techniques will be discussed in this manuscript. Briefly, the controlled-rate method is a "time-consuming" process that requires specific equipment and high-level technical expertise. The second one is a less expensive freezing technique since it does not require a computer-controlled device. However, there are reports that controlled-rate freezing is more effective (compared to the uncontrolled-rate technique) because of higher post-thaw cellular quantitative/qualitative recovery [5, 16–22].

The "osmotic threshold" vs. crystallization or vitrification

Ice formation (crystallization) is regularly initiated by a nucleation (homogeneous or heterogeneous) process. It is carried out by hydrophilic sites on a particle that "mimics" the water molecule collections on the surface of an ice crystal [4]. Different fluids rarely or almost never freeze spontaneously at the melting temperature due to the lack of sufficiently large "nucleators". Unfrozen liquids at a temperature below their nominal freezing point (such as 0 °C for water) are called "super-cooled" fluids. Regardless of the fact whether

nucleation is homogeneous or heterogeneous, the presence of solutes in each solution decreases the fluid's freezing point/temperature. Otherwise, it is a critical fact that functioning living cells do not naturally have ice nucleators in their intracellular organization or area. Hence, they can get "super-cooled" close to temperatures of -30 ºC or even -40 °C when homogeneous nucleation becomes evident [3–5]. Since cells do not normally contain ice nucleators, initially, extracellular ice develops, especially during relatively slow cooling [4, 5].

Cells have a limit to their stability in isotonic conditions (hyperosmotic or hypoosmotic restriction). Cell exposure to hyperosmotic conditions results in reversible or irreversible alterations of membrane permeability and integrity ("membrane stress") while rarely leading to the extrusion of some membrane components. However, cells have a limit to swelling in a hypoosmotic environment [4, 5].

During freezing by slow CS, significant ice crystals are formed in the extracellular space. These crystals do not regularly initiate/create mechanical cell cryoinjury (transmembrane penetration) despite their physical existence. However, the formation of ice crystals in the extracellular area causes a permanent osmotic gradient rise with subsequent intracellular fluid (water) efflux. As a final consequence, cells become dehydrated (volume reduction), followed by resulting organelle and membrane malformations, as well as complete cell destruction or cytolysis [4–11].

At rapid cell freezing, extracellular vs. intracellular osmotic gradient has no time to arise/intensify; consequently, cell dehydration and volume reduction are minor or not observed. Intracellular ice crystal development and enlargement following mechanical cell injury is the most damaging event. The level of cell damage correlates with the whole intracellular ice mass and the size of solitary ice crystals [5–8]. The process of intracellular ice crystallization occurs in the following situations: 1) when a super-cooled cytoplasm approaches the temperature specific for nucleation and freezes spontaneously; 2) while the diameter of ice particles, which is getting smaller as the medium temperature decreases, becomes so small that it can penetrate membrane pores; 3) even when membrane damages allow ice to grow through cell membrane defects. Once ice forms inside cells by crystallization, it can result in mechanical damage to cellular organelles/structures, as well as cell membranes [4, 5].

As stated, crystallization could be also prevented by lowering the freezing point when raising the solute concentration in a solution. Due to the rapid cooling of liquid solution (in the absence of nucleators and crystallization), a specific process can develop a process known as vitrification. It is a biophysical/biochemical process of transition of some liquid solution into an amorphous glass. As the temperature decreases, solutions gradually become more viscous. Precisely, synchronized co-action of growth of solute concentration and decrease of temperature increases the viscosity of the medium in the unfrozen residual solution and reduces the speed and rate at which water can move from a liquid solution to the structure of ice crystals. Thus, at a sufficiently low temperature and at a high enough solute concentration, the solution becomes a solid glass substance (vitrification). Since the nucleating rate is minor at extremely low temperatures, it is possible to prevent the nucleation process and ice crystallization during an ultra-rapid cooling of solutions, as well as obtain a vitrification process in the absence of solutes [4, 5].

Therefore, the determination of an optimized freezing approach and CR (specific for each "cryo-biosystem") should be considered. It is a sufficiently high CS that prevents cell dehydration, as well as adequately low for the efflux of water from cells (preventing intracellular ice formation). The optimized CR during CP can be defined with the ratio of cell surface/volume by the permeability of the membrane for water and other substances, as well as its specific temperature coefficient; however, it also depends on which freezing technique is applied [4, 5, 11].

Consequently, the basic goal of each freezing protocol is to avoid intracellular crystallization and make real intracellular vitrification. The correlation between cell damage from extracellular vs. intracellular ice crystallization, as a function (among others) of CR, was determined and established in initial cryo-investigations [5–10]. Possible causative mechanisms of non-crystallization-mediated cell thermal damages incorporate the concentration of intracellular solutes (salts and sugars) and the occurrence of the "membrane stress" followed by cell volume reduction. The category and intensity of undesired alterations owing to cell "membrane stress" is dependent on the cell type, the temperature, and the category/concentration of extracellular solutes [5, 11–20].

During the use of controlled-rate freezing, if the released fusion heat is not considered and not compensated, it could result in additional temperature fluctuation in the "cryo-biosystem" with further cellular thermal damage. In other words, most studies recommend 1 °C/min as an optimized CS for SCs and platelet CP, although there are reports that these CRs are perhaps higher (nearly 2–3 °C/min) [5, 16, 22–26]. The "phase transition" step of freezing is also critical because a significant reduction in cell recovery/viability was detected when this step was elongated. Thus, the optimal CR for CP of blood-derived cells mentioned above is 1 ºC/min, with a superior CS (2 ºC/min) at "phase transition" step [5, 17–20, 26]. Finally, there are reports that uncontrolled-rate systems can be also effective for SCs and platelet cryostorage [19, 20, 27–31]. However, this system could generate an unbalanced freezing process (CR $\geq$ 3 ºC/min). Therefore, the configuration of "freezing bags" and the volume of cell suspension ("bag-thickness") are also hazardous parameters, which could significantly change the freezing procedure (the kinetics of programmed CR) [5].

Last but not least, cryoinjuries may also develop due to "dilution shock" or cell "swelling", as well as ice recrystallization during thawing [5, 23–25]. Rapid and massive ice thawing produces extracellular water mass increase with a following hypoosmotic condition. As a result of extracellular hypoosmolality, besides minor effusion of penetrating CPt from cells, a massive water influx into the intracellular space happens following the "dilution shock". Cells are usually more vulnerable to enlargement than to the reduction of their volume; consequently, they can be simply destroyed due to the

"dilution shock" [4, 5]. Throughout extracellular recrystallization, additional cell dehydration could happen. Again, during intracellular recrystallization, further mechanical cell damage can be expressed; small ice crystals could develop into crystal agglomerates or enlarge their mass [4–11].

Cryoprotective agents – types and working options

The choice and use of a high-class freezing technique is essential, but it cannot explain cryoinjury origin nor eliminate it completely. Post-thaw cell recovery/viability is superior merely when effective CPt is added to the "cryobiosystem" to prevent/reduce potential thermal damages. Mechanisms of the action of various CPt are complex and incompletely explained. Due to the differences in their physicochemical/biophysical properties, it is not possible to determine a general protective mechanism for them all [5].

CAs can be categorized into intracellular or penetrating and extracellular or nonpenetrating compounds. The potential/speed for "trans-membrane penetration" of CPt is very important for successful cell protection. However, cryoprotection can be achieved by quickly penetrating agents (DMSO) and slowly penetrating agents (glycerol), as well as by nonpenetrating agents (HES). Typically, intracellular CAs could give cell protection during low-rate freezing by decreasing the intensity of cellular dehydration and volume reduction. Then again, extracellular CPt could protect cells commonly during rapid freezing, reducing the degree of intracellular ice formation [3–5]. The speed and quantity of CPt trans-membrane penetration (diffusion or influx) is, in fact, again a temperature-dependent process. Namely, the temperature of the suspension at which the cell is exposed to a CPt has also an effect on diffusion rate; at lower temperature levels, poorer CPt influx/efflux was observed [1–5].

The use of CAs in sufficiently high concentration but below critical cytotoxicity (especially DMSO) and temperature fall results in intracellular hyperviscosity. Because of that, water molecule mobility and subsequent crystallization are delayed. Penetrating CPt increases intracellular solute concentration, producing a condition with a lower temperature at which ice crystallization will develop. These agents and events also reduce extracellular vs. intracellular osmotic gradient (minimized "solution effect"). Finally, certain CPt can simply modify water trans-membrane penetration rate, thus affecting the level of cell dehydration [5, 7–9, 11–13].

As stated, glycerol and DMSO were discovered in the middle of the last century for CP of blood-derived and other cells. As main penetrating CPt, they have superior molar volume than low MW intracellular salts and sugars (e.g., the volume of one mole of glycerol and NaCl are 40.7 mL and 27 mL, respectively) [4]. Hence, their use could delay and diminish cell dehydration and volume reduction even at low temperatures. Besides the effects on osmotic gradient, glycerol and DMSO have additional effects. Namely, the protective action of these intracellular CAs is achieved because of their colligative (connective) effect, that is, the potential for water binding [4, 5].

Glycerol, DMSO, and HES show particular effectiveness in blood-derived cell CP. They are key hydrogen bond acceptors; consequently, they can effectively connect a high quantity of water molecules, and, as a result, they have an important cryoprotective potential. For that reason, cells can be stored under a nominal freezing point of a specific solution with no extreme intracellular ice crystallization and severe dehydration [5].

Concisely, DMSO could be described as a transparent or colorless fluid with a sulfur-like smell. DMSO is a very polar molecule that dissolves many water-soluble and lipid-soluble substances. It has exothermic properties and should be mixed slowly with the cell suspension to dissipate the generated heat. Furthermore, given intravenously (even in small concentrations), DMSO may cause certain adverse events such as nausea, vomiting, local vasospasm, etc. [7–9, 11–13].

Very important nonpenetrating CA is the HES with average MW ranging from high (≥ 450 kDa), medium (200–400 kDa) to low MW (150–200 kDa). Therefore, it has a larger MW than glycerol or DMSO. Combined with DMSO, HES was originally used for granulocyte CP. As a potent cryoprotector, HES acts predominantly extracellularly during low-rate freezing [5, 21].

The action of cryoprotection using HES is different from that for penetrating cryoprotectors such as glycerol and DMSO, which reduce the solution freezing point and decrease the temperature at which the salt concentration becomes cell-destructive [5, 21]. Namely, the cryoprotective action of HES is, above all, the result of its ability to absorb water molecules (around 0.5 g of water per 1 g of HES) and keep these molecules thermally "inert" in a glassy state missing event of "phase transition" (liquid into solid stage) during (super)cooling. Thus, HES affects the viscosity of the solution and reduces the CR required for cell survival during vitrification, reducing/delaying the ice formation [3–5, 21]. Finally, there is data that CP of blood-derived cells using HES with DMSO is possible (cells frozen by this technique have sufficient post-thaw recovery/viability) [5, 11–13].

In summary, for CP of SCs, progenitors, different white blood cells (lymphocyte and granulocyte freezing systems), and platelets (whose freezing represents a specific challenge due to their limited tolerance to osmotic fluctuations), typically DMSO or DMSO with HES, are used as effective CAs, although in various final concentrations and/or combinations. They can express a protective effect due to the reduction of cellular dehydration and/or a decrease in the number of intracellular ice crystals. However, CAs cannot protect cells from dehydration that already exists or from the effects of earlier-developed ice crystals in the intracellular space [2–5].

From stemness to cryopreserved cell practice – experimental and clinical data

Hematopoietic tissue was the first and most explored cytopoietic or "tissue-generating" system in humans. Stemness is a (hemo)biological molecular process that combines the ability of an immature cell to maintain or perpetuate its lineage (self-renewal or self-maintenance capacity), give rise

to several more developed daughter cells or specialized mature cells (differentiation and proliferation potential), and interact with milieu, extracellular matrix to continue and keep a balance between cellular latency, proliferation, and regeneration. The very primitive SCs compartment could be illustrated as cells with high-pitched expression of developmental pathways and by the important intensity of epigenetic plasticity [5, 32–37]. In the steady state circumstance, the character and actions of SCs are regulated with a set of genes and by well-organized and precisely synchronized signaling systems. Defects in signaling cascade(s) or loss of intercellular balance (cell-cell communication) can initiate uncontrolled cell growth or death, as well as cell malfunctions and/or transformation (development into a variety of diseases, including tissue defects or cancer) [23–25, 35–40]. Together with explained physicochemical/biophysical factors, SC-related events are also regulated and restricted by the mechanical environment in which SCs reside and stay alive. The process of SCs-biology "supervising" through specific mechanical factors remains inadequately understood or still lacking, and it is the strategic target for developing the field of mechanobiology [41, 42].

The existence and functioning of SC partition guarantee steady-state homeostasis in each tissue-generating system. Hematopoietic SCs are capable and competent to provide bone marrow (BM) repopulation following SCs-transplant in patients with partially or completely damaged hematopoiesis and some other disorders. The compartment of SCs and progenitors express a specific CD34 antigen. Thus, they are also called $CD34^+$ cells, a cluster differentiation/designation (CD) marker for a transmembrane glycoprotein. On the cell membrane of more primitive SCs, the CD90 antigen (a specific marker for more immature $CD34^+/CD90^+$ compartment or repopulating SCs) is also inherent. The occurrence of cells expressing this antigen in the graft is essential for complete, stable, and long-term marrow repopulation following SCs-transplant with hematopoietic reconstitution [5, 23, 26].

Generally, SCs can be collected from BM using multiple aspirations or with harvesting from peripheral blood (PB) after mobilization and by processing (purification) of umbilical cord blood. For therapeutic use (SCs-transplants or regenerative medicine), BM was the first SCs source. Cells are collected from the posterior and anterior iliac crest (rarely from the sternum). The optimal timing for allogeneic PB-derived SCs harvesting is on the fifth day (at maximum "$CD34^+$ peak") of recombinant human granulocyte colony-stimulating factor (rHuG-CSF) administration. However, determining the optimal timing for autologous SC harvesting is more complex. These patients are given higher rHuG-CSF doses combined with chemotherapy. The count of circulating $CD34^+$ cells correlates with the superior $CD34^+$ yield in the harvest. When the number of $CD34^+ \geq 40/\mu L$ in PB, the possibility of collecting $CD34^+ \geq 2.5 \times 10^6$/kg/body mass (bm), or more, is approximately 60% [5, 30].

An innovative SCs mobilizing regimen uses plerixafor to obtain adequate SCs yield from the blood of "poor responders" ("poor mobilizers") [5, 30, 43]. Our data also confirmed the efficacy of this mobilization protocol using plerixafor (combined with rHuG-CSF); the $CD34^+$ count was higher in PB, and cell yield was superior in the harvest [23–25, 30]. A successful SC transplant can be expected when the yield of $CD34^+$ cells is $2–4 \times 10^6$/kg/bm (or more likely $\geq 5 \times 10^6$/kg/bm) [5, 30]. Finally, our preclinical SCs cryoinvestigation has confirmed that the ratio of more primitive SCs ($CD34^+/CD90^+$ subset) in PB could also be a useful mobilization predictive factor to determine optimized timing for cell harvesting and predictor of the quality of harvest [24–26].

However, the use of an effective SC transplant requires both high-quality collection/harvesting methods and CP systems to obtain an adequate cell yield, as well as quantitative/qualitative cell recovery. In practice, CP of BM-derived SCs incorporates the following steps: 1) marrow aspirate processing (pre-freezing depletion of red blood cells and plasma); 2) cell exposure to a freezing medium with CA (equilibration); 3) freezing the mixture of cells in medium; 4) cryostorage at -130 ± 10 °C (mechanical freezer or nitrogen steam) or at -196 °C (liquid nitrogen); 5) thawing in a water bath at temperature 37 ± 3 °C.

The PB-derived SCs CP should be modified, or in other words, adapted to conditions that depend on superior mononuclear cell number, the presence of proteins (albumin) in the plasma, and the lack of lipid or bone particles in the harvest. Following the thawing procedure, SCs are immediately applied (reinfused) across a central venous catheter to the patient. Recipients tolerate well this reinfusion, lacking DMSO-related adverse effects. The incidence of potential reinfusion-associated side effects (typically nausea and vomiting) is regularly a function of the DMSO concentration (quantity) in thawed cell suspension [5, 4–9, 16, 22, 23]. In this context, there are reports that the use of lower DMSO concentration (5%) rather than higher (10%) results in a superior $CD34^+$ recovery (inferior apoptotic and necrotic $CD34^+$ cell incidence) with an elevated engraftment potential of these cells [31]. At last, there are data that the concentrations of DMSO from 2.2% to 3.5% are also adequately qualified for a satisfactory cell recovery following SCs transplant [29].

Finally, let us summarize our activities in cryo-practice using primarily controlled-rate freezing systems (with fusion-heat compensation) vs. uncontrolled-rate method ("dump-freezing") in experimental or (pre)clinical settings. Our experimental and (pre)clinical results are comparable with data from the literature, as well as above cited studies [5, 17–20, 30].

In an experimental setting, we have found that the recovery/clonogenicity of less primitive SCs populations (pluripotent and committed progenitors: CFU-Sd12 and CFU-GM) was higher in the presence of 5% vs. 10% of DMSO. On the contrary, it has been verified that the recovery/viability of very primitive SCs (marrow-repopulating ability cells) was superior when 10% of DMSO was applied. These results mean a different cryobiological "request" of marrow-repopulating ability cells vs. more mature progenitors. We have demonstrated in these experimental studies that deviations in cell recovery, clonogenicity, and viability are significantly related to the cell-specific CP strategy used (freezing technique with appropriate DMSO concentration) [5, 17].

Briefly, SC transplants were used for the treatment of our patients with acute lymphoblastic leukemia and non-lymphoblastic leukemia, chronic myeloid leukemia, multiple myeloma (MM), Hodgkin lymphoma and non-Hodgkin lymphoma, as well as patients with severe aplastic anemia, and multiple sclerosis [5, 23–25, 30]. Mobilization of SCs was accomplished with rHuG-CSF (by standard dose, 12–16 μg/kg/bm) following chemotherapy, for instance, by salvage (platinum-based) regimen (lymphoma patients), as well as applying a poly-chemotherapy pre-treatment (cyclophosphamide, adriamycin and dexamethasone) or using high dose cyclophosphamide (MM patients). For "poor mobilizers", the second mobilization using rHuG-CSF (16 μg/kg/bm) with plerixafor (24 to 48 mg around 6–11 hrs prior to cell collection) was completed ($CD34^+$ cell yield $\geq 4 \times 10^6$/kg/bm in the harvest) [30]. The SCs harvesting (by Cobe®-Spectra or Spectra-Optia®; Terumo-BCT, USA) was initiated only at the "cut-off" value of circulating $CD34^+$ cells, at 20×10^6/L or more [5, 30]. Harvested cells were frozen by our original controlled-rate freezing procedure using an optimized DMSO concentration (final DMSO concentration of 10%) and stored at -130 ± 10 ºC (mechanical freezer) or at -196 ºC (liquid nitrogen) and thawed directly prior to the clinical application (using water bath at 37 ± 3 °C) [5, 30]. Hematopoietic reconstitution was rapid – the average time for neutrophil recovery was on the 12th day (range 6–26 days), and average platelet recovery was on the 12th day (range 5–44 days) [5, 15, 23–25, 30]. The overall SC transplant efficacy was dependent on the type, stage, and chemosensitivity of the disease, presence of co-morbidities, general health status, and age of the patient, as well as the degree of human leukocyte antigens-HLA matching [5, 14–16, 30].

Lastly, our preclinical results demonstrated that platelet recovery was superior when the strictly equalized six-step controlled-rate freezing (CR = 1 °C/min), with compensation of the released fusion heat (CR = 2 °C/min) during the "phase transition" period, in combination with lower DMSO concentration (6% in autologous plasma), was used. Only minor intergroup differences (between protocols) for parameters of cell recovery, integrity, and functionality were observed [19, 20].

Conclusion

The increased use of myeloablative treatments (combined with SCs rescue), as well as intensified application of cell-mediated therapies, has resulted in superior requirements for both the SCs and practical operating procedures to improve cell yield and recovery, as well as minimize cellular damages during harvesting and/or CP.

CP is a well-working system that protects cellular organelles, isolated cells, cell clusters, or tissues during freezing (by ultra-low cooling), long-term cryostorage, and rapid thawing procedures, and it is beneficial in cases where cells are vulnerable and unstable during durable preservation in liquid-state. The major CP requirement is to reduce cell thermal damage ("cryoinjury-score") and its consequences. Existing CP techniques applicable in cryo-practice should be re-evaluated and improved. Further basic research and (pre)clinical cryo-investigations are recommended or required to define well operating "cryo-biosystem" (specific for each cell type) in order to obtain an optimized post-thaw cell recovery and viability.

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