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REVIEW

Post-thaw viability of cryopreserved peripheral blood stem cells: what are we actually looking at?

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SUMMARY

Autologous stem cell transplant is a standard of care for a variety of hematological malignant and non-malignant diseases, and the process of stem cell collection, cryopreservation, thawing and reinfusion, defined as "stem cell processing" has evolved thoroughly in recent years. The enumeration of CD34+ cells within the graft represents a surrogate of its clonogenic potential and is today widely recognized as the main parameter capable of predicting hematopoietic recovery. Post-thaw viability assay is routinely performed as a measure of graft quality for autologous transplant. However, quality control measures are challenging as there is not standardized assay for such test, little is known on the correlation between viable CD34+ count and graft clonogenic potential, and progenitor cells responsible for hematopoietic recovery are actually only a minority of the hematopoietic stem cells infused within the peripheral blood graft. We will herein briefly review the main issues on post-thaw hematopoietic stem cell viability test, with the aim to provide practical answers to some of the main open questions on this topic.

INTRODUCTION

Autologous stem cell transplantat is a standard of care for a variety of hematological malignant and non-malignant diseases. The process of stem cell collection, cryopreservation, thawing and reinfusion, defined as "stem cell processing" has evolved thoroughly in recent years (1). Initial studies carried out in the late 1950's employed bone marrow stem cells stored at room temperature while the conditioning chemotherapy was infused (2). More recently, mobilized peripheral blood stem cells (PBSC) became the preferred graft source for autologous transplant (3) allowing longer interval between collection and transplant, faster hematopoietic recovery, reduced morbidity and mortality, and inferior risk of graft contamination by tumor cells (4).

The success of autologous transplant is mainly based on the ability of PBSC graft to rapidly reconstitute normal

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hematopoiesis. The enumeration of CD34+ cells within the graft represents a surrogate of its clonogenic potential and is today widely recognized as the main parameter capable of predicting hematopoietic recovery. The minimal dose of CD34+ cells to be infused in order to obtain sustained engraftment has not been defined yet; however, a threshold of $2x10^6$/kg CD34+ is commonly accepted as the minimum amount of stem cells capable of providing stable 3-lineage hematopoietic recovery (5, 6).

CD34+ cell enumeration is routinely performed on the fresh graft at the time of harvest, and little is known on the detrimental effect that cryopreservation and thawing processes impose on stem cell viability and functionality. In fact, the number of viable and fully functional HSCs infused to the patient is invariably lower than that enumerated at the time of harvest (7, 8), and the assessment of the extent of loss of viable HSCs during stem cell processing remains challenging.

Viability and potency of the stem cell product constitute formal release criteria from cryopreservation facilities; in fact, since the infused stem cells are, by definition, a cell therapy, rigorous quality parameters and control measures are required by regulatory agencies (9). Further, those parameters hold heavy clinical implications, since are strictly related to transplant safety and patient outcome.

Post-thaw viability assay is routinely performed as a measure of graft quality for autologous transplant. Enumeration of viable CD34+ cells at the time of infusion becomes particularly relevant in patients in whom stem cell mobilization has been problematic and/or collected a total amount of stem cells which is borderline to ensure a safe transplant procedure (i.e. $2x10^6$ CD34+/kg). However, quality control measures are challenging as there is not standardized assay for such test, little is known on the correlation between viable CD34+ count and graft clonogenic potential, and progenitor cells responsible for hematopoietic recovery are actually only a minority of the hematopoietic stem cells infused within the peripheral blood graft.

We will herein briefly review the main issues on post-thaw hematopoietic stem cell viability test, with the aim to provide practical answers to some of the main open questions on this topic.

Stem cell processing and post-thaw CD34+ viability

As previously stated, 2×10^6 viable CD34+ cells/kg enumerated at the time of harvest is widely accepted as the minimum requirement for a sustained 3-lineage engraftment; it is however unclear what should be considered as the minimum threshold when CD34+ cell dose is assessed after cryopreservation.

Within stem cell processing, the most critical phases which impose the highest stress on stem cells include:

1. the pre-freezing phase;
2. the freezing process;
3. thawing and infusion delay after thawing.

Regarding the pre-freezing phase, factors which have been showed to negatively affect stem cell viability include the time interval between harvest and cryopreservation and graft contamination by white blood cells (WBC) (10-13).

Cryopreservation process is a com-

plex and delicate phase; in fact, as the cells cool from room temperature to the freeze point, ice crystals forming in extracellular and intracellular medium impose a great osmotic stress on the hematopoietic stem cell. Different studies compared passive (uncontrolled) freezing with the more recently introduced controlled freezing method, providing conflicting evidence regarding their effect on CD34+ viability (14, 15).

Uncontrolled freezing has been employed for many years and is still routinely used by many centers; the main disadvantage of this method is that the cooling process is fixed and not modifiable. The more recent introduction of controlled freezing techniques allowed for a customization of the cooling curve and a finer control of the freezing process. However, there is no single standard freezing profile yet, and some authors found a detrimental effect on CD34+ viability of an excessively fast cooling (faster than 6°C/min), and engraftment delays have been reported (16).

For what concerns the post-thawing phase, clinicians are highly worried in the everyday practice by occurrence of delays in stem cell reinfusion after thawing; however, a significant body of evidence suggests that re-infusion can occur within 120 minutes from thawing without a significant loss in stem cell viability; nevertheless the majority of centers agree on a maximum accepted delay of 30 minutes.

Post-thaw CD34+ viability and engraftment kinetics

Different authors suggested that post-thaw assessment of CD34+ cell viability may be a more accurate method to predict hematopoietic engraftment rather than CD34+ cell enumeration at the time of harvest.

Allan et al. (17) analyzed 36 patients with different diseases showing a correlation between post-thaw viability and platelet recovery, while no association was observed with neutrophil engraftment. Similarly in a study by the Australian group (18) the effect of a post-thaw loss in CD34+ cell viability was associated with a delay in platelet but not neutrophil engraftment.

Conversely, Yang et al. (19) found, in a population of 52 patients with mixed diseases, a correlation between post-thaw viable CD34+/kg and both neutrophil and platelets recovery; interestingly, the authors further observed an association between viable CD34+/kg and colony forming unit (CFU)-GM. Similar results were provided by a Korean study (20).

The fact that platelet recovery seems to be more dependent on viable CD34+ cell count as compared to neutrophil engraftment might be partially explained by two main reasons:

- neutrophil recovery has a limited inter-patient variability and occurs in most patients within day 10 and day 15 after transplantation;
- the administration of G-CSF after transplant probably contributes to further narrowing this range, while inter-patient variability in platelet recovery is significantly wider.

Interestingly, the extent of CD34+ stem cell loss from the time of harvest to the re-infused graft was about 25-35% in most of the cited studies; this piece of data should be highlighted, as CD34+ threshold determining a "safe" transplant is commonly defined using the pre-cryopreservation CD34+ count,

TABLE 1 • Studies analyzing correlation between post-thaw viable CD34+ and engraftment.

Author	Diseases	No. of patients	Correlation with engraftment		CD34+ loss at thawing
			Neutrophils	Platelets	
Allan (17)	MM, NHL, HL, GCT	36	No	Yes	45%
Yang (18)	MM, NHL, HL, Amyloidosis, ST	52	Yes	Yes	34%
Lee (20)	MM, NHL, HL, AL, ST	36	Yes	Yes	27%
D'Rozario (18)	MM, NHL, HL, Amyloidosis, GCT, MS	106	No	Yes	33%

List of abbreviations: GCT, Germ cell tumor; HL, Hodgkin Lymphoma; MM, Multiple Myeloma; MS, Multiple Sclerosis; NHL, Non-Hodgkin Lymphoma; ST, Solid tumors.

rather than the one assessed after thawing (Table 1).

Post-thaw CD34+ viability and transplant safety and efficiency

Many efforts have been done to find an association between the amount of viable CD34+ cells infused to the patients and transplant safety and efficiency. Most authors and clinician would agree that performing an autologous stem cell transplantation with at least $2x10^6$/kg viable CD34+ can be considered safe. However, a lower CD34+ cell dose might be sufficient if colony forming units (CFU) are at least $2x10^5$, as proposed by Watts (21). Interestingly, in this study a dose of only $1x10^6$ CD34+/kg was associated with satisfactory engraftment if this stem cell dose was part of a greater collection, while it correlated with delayed recovery if that was the total amount of stem cells collected for that given patient.

Nevertheless, neutrophil and platelet recovery are imperfect parameters to test graft quality and transplant efficiency.

Rozario et al. (18) observed a strong association between viable CD34+ infused and outcome measures as need of red cell and platelet transfusions, G-CSF and IV antibiotic administration, and length of patients' ospitalization.

Similarly, in a recent EBMT study coordinated by Lanza (22) conducted on almost 400 patients, the efficiency of the autograft procedure was analyzed with regard to complex clinical endpoints; in fact, outcome measures for graft quality and efficiency of the transplant were defined as health economic efficacy (eg, antibiotic administration, transfusion of blood components, and time in hospital), toxicity (in accordance with Common Toxicity Criteria), and safety (i.e. the risk of regimen-related death or disease progression within the first year after PBSCT). A time-dependent grading of efficacy was proposed with day 21 for multiple myeloma and day 25 for the other disease categories (depending on the length of the conditioning regimen) as the acceptable maximum time in hospital, which together with antibiotics, antifungal, or transfusion therapy delineates four groups: favorable (7 days on antibiotics and no transfusions; 21 [25] days in hospital), intermediate (from 7 to 10 days on antibiotics and

<3 transfusions, 21 to 25 days in hospital or 7 days on antibiotics and no transfusions; from 21 to 30 days [25 to 34] in hospital), unfavorable (>7 days on antibiotics, >3 but <6 transfusions; >30/34 days in hospital after transplantation), and very unfavorable (>10 days on antibiotics, >6 transfusions; >30 to 34 days in hospital) (Table 2). Interestingly, factors which emerged as predictors of favourable outcome were a stem cell harvest of >=4x10^6/kg CD34+ in 1 apheresis and an inferior graft volume reinfused (<500 ml). It should be highlighted that only a minority of transplants resulted as "efficacious" as defined by the criteria proposed by the authors.

Is there a standard technique to evaluate post-thaw CD34+ viability?

Techniques for enumerating viable CD34+ cells in the PBSC graft have greatly developed in recent years, evolving from trypan blue assay to multiparametric flow cytometry (MPFC) (23), which is considered today the standard technique for CD34+ viability testing in fresh and thawed graft sample. In early 1995, the International Society of Hematotherapy and Graft Engineering (ISHAGE) developed a protocol which is today widely accepted as standard gating strategy for CD34+ cell enumeration in flow cytometry (24). This assay, combined with the viability stain 7-aminoactinomicin D (7-AAD) is routinely employed at the time of harvest to enumerate the viable CD34+ cells within the graft. The ISHAGE-MPFC/7-AAD represents the best surrogate of CFU assay, which still remains the most reliable test for evaluating stem cell functionality and clonogenic potential; however this "old-fashioned" assay holds major dis-

TABLE 2 • Clinical endpoints of transplant efficiency as defined by Lanza (22).

Objectives	End points		Grading
Primary	To study the efficacy of stem cell graft reinfusion	Days of hospitalisation, interventional antibiotic and antifungal treatment and transfusion of blood components	• Acceptable: ≤21* days in hospital and no transfusions • Not acceptable: >21 days in hospital or on continuous antibiotic or antifungal treatment
Secondary	To evaluate toxicity following stem cell graft reinfusion	Common Toxicity (CTC) as defined by WHO or derived references (ECOG, SWOG etc.) for mucositis, dermatitis and enteritis as well as grading of haematological toxicity	• Acceptable: toxicity grade 0-1 • Not acceptable: toxicity grade 3-4 • For haematological toxicity: See ref 1 in Synopsis.
Tertiary	To evaluate safety following stem cell graft reinfusion	Regimen-related death or disease recurrence	• Acceptable: alive and in complete remission day 100 • Not acceptable: death or disease progression

advantages, being time-consuming and not standardized, and is therefore very rarely performed today in clinical practice.

Nevertheless, ISHAGE-MPFC/7-AAD technique presents several limits itself. First of all, this test was originally developed for analyzing fresh samples, and it is currently not standardized in cryopreserved and thawed graft. Further, 7-AAD staining is unable to detect pre-apoptotic stem cells, which are hence considered viable by the test but actually unable to form colonies and engraft (25). Those limits emerged in the context of cord blood transplant, where different centers found a significant discrepancy between the CD34+ cell content of the CBU unit as reported by the cord blood bank and that observed by transplant center at the time of re-infusion. Basing on those data, some authors have raised the concern that ISHAGE assay might be inaccurate for post-thaw CD34+ viability testing, as a significant proportion of non viable CD34+ remains undetected, hence leading to an overestimation of CD34+ viability.

Basing on this hypothesis Saccardi, in collaboration with the major European cord blood banks, recently proposed a modification of the standard ISHAGE (SI) gating strategy with the aim of obtaining a more reliable post-thaw CD34+ enumeration in cryopreserved CBU units (26). The main innovation consists in the enlargement of the lymphomonocytic gate towards the axes intercept in order to include in the count the non viable CD34+ cells which escape SI detection. The new technique (modified ISHAGE, MI) provided similar results in the fresh samples when compared to SI; however, in the cryopreserved graft MI resulted in a higher recovery of CD34+ cells, while similar number of viable cells were de-

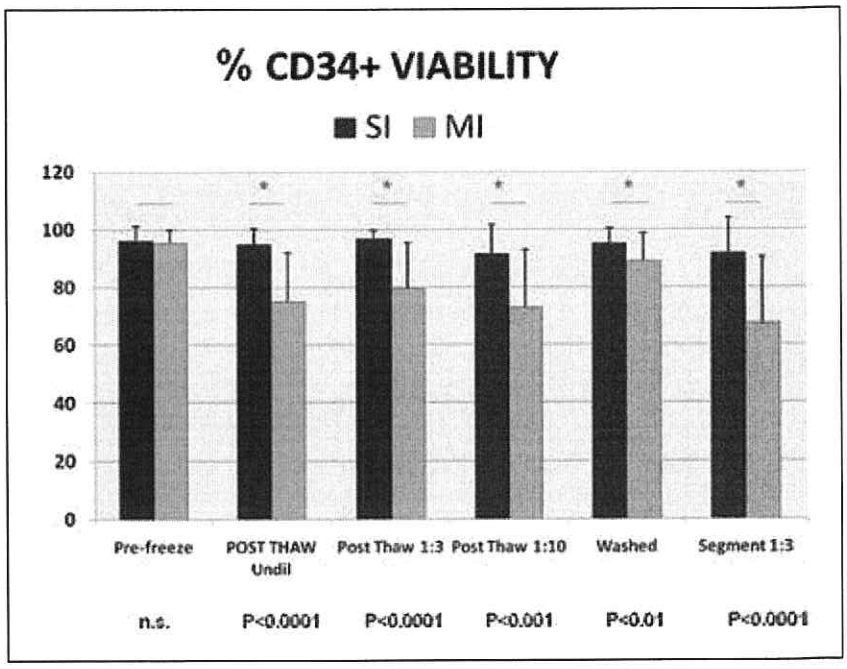

FIGURE 1 • Pre-freeze and post-thaw viability assessed by Standard and Modified ISHAGE in samples prepared with different techniques (Saccardi et al., 26).

tected, thus obtaining a viability percentage which was inferior to that reported with SI (Figure 1). Interestingly, MI showed a better correlation with CFU assay as compared to SI.
Subsequently, Lanza et al. (unpublished) tested MI on 75 autologous graft collected and stored at Cremona Hospital, obtaining consistent results. These data confirmed the validity of the modified ISHAGE protocol for the accurate enumeration of CD34+ cells in cryopreserved material, allowing a better assessment of stem cell recovery compared with that of standard ISHAGE.
Finally, stem cell viability does not match with stem cell functionality. As previously stated, CFU remains today the only assay capable of testing the clonogenic potential of stem cells infused to the patients. Shoulars (27) recently proposed a rapid MPFC technique which enumerates cells expressing high levels of the enzyme aldehyde dehydrogenase along with viable CD45+ or CD34+ cell content; interestingly, the authors found a significant correlation between CD34+ ALDH^{br} count and CFU in about 4000 cord blood units. This appears a promising approach to rapidly test graft potency and quality.

CD34+ cell subset analysis
The assessment of CD34 cell subset composition in PBSC and marrow may allow a more mature definition of minimum graft size, as the precise phenotypes of the HSCs responsible for short and long-term engraftment are still controversial. These studies will be of importance not only for patients who mobilise their HPC poorly, but also for optimal utilisation of resources in the general settings of hematopoietic stem cell transplant (28). In a recent report, the European Working Group of Clinical Cell Analysis (EWGCCA) has demonstrated that using a standardized, "state of the art" single platform CD34+ stem cell flow cytometric method both intra- and inter- laboratory coefficients of variation can be reduced to less than 5% and 10% respectively (29).
Continuous education and targeted training of individual laboratories form a critical component of this improvement and hopefully will improve the intra- and inter-laboratory variation of CD34 cell subsets determination in the next future. This is a pre-requisite to achieve meaningful multi-centre clinical study data.
The technical issue which remains still unresolved for the analysis of fresh sample is: lyse, no-wash vs no lyse, no wash sample preparations, while for cryopreserved/thawed samples an ISHAGE derived protocol should be extended to the simultaneous analysis of stem cell viability as absolute count.
On the other hand, the clinical issue to address is: could CD34 subsetting represent an implementation of the graft potential? (30, 31). To answer this question several actions should be taken:

- The evaluation of existing protocols in order to improve them or to create new ones as needed. The more complex protocol to elaborate will be the simultaneous subset analysis and viability assay that will allow subset characterisation of living HSCs at the time of infusion. This study trial will be performed through the consecutive control of the fresh sample, a cryopreserved and thawed reference ampoule and

the cryopreserved thawed graft to be infused to the patient.

- The selection of reference standards for standardisation trials: the more a protocol is sophisticated, the greater the need for monitoring the protocol and its use.
- As the MPFC analysis of the different CD34+ cell subsets obtained from leukapheresis products is far from being standardized, a feasibility study has to be undertaken, with the aim of evaluating whether a reliable enumeration of absolute numbers of CD34+ subsets can be achieved in a multicentre study. This analysis may provide useful information for a better definition of the minimum amount of stem cells required for a successful transplantation of cryopreserved PBSC.

CONCLUSIONS

Post-thaw viability of cryopreserved PBSC is a quality control measure which holds major clinical implications; however it appears still far to be reliable and standardized. From collection to reinfusion HSCs pass through different manipulation steps which unavoidably affect graft quality and stem cell functionality. The main factors recognized to affect cryopreserved HSCs viability are latency time between stem cell collection and cryopreservation, WBC contamination of the graft, method of cryopreservation (passive or controlled freezing), freezing speed and, after thawing, delay in HSCs reinfusion to the patient. There appears to be some kind of correlation between viable CD34+ cells infused and engraftment, mostly with platelet recovery; however more complex outcome measures beyond hematopoietic recovery should be probably considered. While the ISHAGE/7-AAD platform can be considered today a standard for viable CD34+ enumeration in the fresh graft, that does not hold true for the cryopreserved product. Further, viable CD34+ cell tests are probably inaccurate in assessing stem cell function after cryopreservation, and there is a need for alternative, rapid, reliable and reproducible functional assays to replace CFU test. Finally, the analysis of CD34+ cell subsets within the apheresis product may allow a finer definition of graft quality; prospective, multicenter studies exploring this issues are warranted.

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