Analysis of Flow Cytometric Enumeration of CD34+ cells in Leukapheresis Product from South Indian Cancer Centre

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Submitted: July 18, 2018; Revised: Feb 18, 2019; Accepted: Apr 8, 2019; Published: Apr 30, 2019

Abstract: Background: The success of peripheral blood stem cell (PBSC) transplant often relies on CD34+ cells counting. As the assay needs to be repeated in duplicate, this study was conducted to observe the variations between the duplicates with respect to concentration of leukapheresis product. Methods: The assay followed was single platform method - ISHAGE protocol published by the International Society of Haematotherapy and Graft Engineering. New parameter named variation index was used to compare the difference between the duplicates. Results: We present our data on 111 samples collected from leukapheresis product. 10% of the assay did not meet the criteria and required repeat testing. We observed no significant difference between the duplicate samples with respect to low (CD34+ cells/µl<500; P=0.632), Medium: 500 to 1000 CD34+ cells/µl. P=0.085 and High >1000 CD34+ cells/µl. P=0.864. Conclusion: Testing the CD34 enumeration in duplicate and verifying its repeatability for variation index of less than 10 will help in accurately determining CD34+ dose calculations.

Key Words: CD34+ cells Enumeration, Flowcytometry, ISHAGE

Introduction: Peripheral blood stem cell (PBSC) collection has progressively replaced bone marrow (BM) as the source for hematopoietic progenitor cells (HPC) for both autologous and allogeneic transplant. PBSC collection involves separation and collection of white blood cells enriched for CD34+ HPC a subset of mononuclear cells from whole blood using apheresis systems.(1) Presently CD34+ HPCs are mobilized into peripheral blood after administration of granulocyte colony stimulating factor (G-CSF) alone or in combination with chemokine CXCR antagonist, Perixxafor.(2) For prompt hematopoietic reconstitution, the dose of HPCs infused in terms of CD34+ cells/kg of body weight is the most reliable marker for predicting engraftment time.

Flow cytometry is the method of choice to assess CD34+cell content and dosage calculation in PBSC transplant. For accurate enumeration, appropriate procedures are necessary to minimize pre-analytic and post analytic variables. (3) Early attempt at enumerating CD34+ cells by flow cytometry was Milan/Mulhouse protocol based on the evaluation of CD34+ cells with a low light scatter (SSC), after initial forward vs SSC live gating excluding debris, platelets and erythrocytes.(4) Most centres follow single platform method - ISHAGE protocol published by the International Society of Haematotherapy and Graft Engineering, designed as a set of guidelines for the accurate detection of CD34+ cells based on four-parameter flowcytometry method(CD45PerCP/CD34 PE staining, side and forward angle light scatter).(5,6) ISHAGE protocol requires samples to be performed in duplicates for repeatability and the mean of the two values should be used for reporting.

The present study was aimed to observe the variation in CD34+ cell count between duplicate samples in leukapheresis product using variation index.

Methods
It was a retrospective study from a tertiary care cancer centre from Jan 2014 to May 2018. Inclusion criteria: Sample aliquoted from PBSC product of both autologous patients and allogenic donors for CD 34 enumeration. Exclusion criteria: Peripheral blood samples collected to know the adequacy of mobilized CD34+ cells before stem cell harvesting was excluded.

The assay was performed in the Department of Transfusion Medicine, using CXP software of FC500 equipment. The CD 34+ cells enumeration was performed on Beckman Coulter FC500 equipment using commercial Stem Kit reagent. ISHAGE guidelines: four-parameter flow cytometry method (CD45FITC/CD34PE staining, side and forward angle light scatter) was used for the enumeration.
Sample preparation
Freshly obtained samples from leukapheresis product with higher WBC concentrations were diluted to 10,000 WBC/μL with phosphate-buffered saline (Table 1). As per ISHAGE protocol, the samples were stained in duplicate (sample 1 & 2) with two colour CD45-FITC/CD34-PE (20μl) monoclonal reagents. A third tube with control reagents (20μl CD45-FITC / IsoClonic Control-PE) was used to check non-specific binding of CD34 antibody. The viability of the cells was checked using 7-AAD Viability Dye (20μl), a nucleic acid dye that binds to accessible base pairs (cellular DNA). Lyse no wash technique was used for red cell lysis using buffered ammonium chloride without washing. Known concentration of 100μl fluorescent beads (polystyrene fluorescent microspheres aqueous suspension medium) was added and thoroughly mixed with the sample just before enumeration.(7)

Table 1: Dilutions used for leukapheresis product with higher WBC concentrations for CD34+ cells enumeration

<table>
<thead>
<tr>
<th>WBC count</th>
<th>Dilutions</th>
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<tbody>
<tr>
<td>&lt;20,000/μL</td>
<td>100 μl blood</td>
</tr>
<tr>
<td>20,000 to 50,000/μL</td>
<td>75 μl blood + 25 μl PBS</td>
</tr>
<tr>
<td>30,000 to 40,000/μL</td>
<td>50 μl blood + 50 μl PBS</td>
</tr>
<tr>
<td>40,000 to 100,000/μL</td>
<td>25 μl blood + 75 μl PBS</td>
</tr>
<tr>
<td>&gt;100,000/μL</td>
<td>10 μl blood + 90 μl PBS</td>
</tr>
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PBS- Phosphate Buffered Saline

Gating
ISHAGE guidelines was followed for estimating absolute number of CD34+cells in single platform (Figure 1).

Calculations
The assay was accepted if the number of CD34+ cells falls within 10% of the mean for the duplicate samples, if falls outside 10%, the assay was repeated. The value obtained with the control tube must represent less than 10% of the average value obtained from the tests tubes to validate the results.

CD34+ HSC Absolute Count (cells / μL) = MeanCD34+ Count (cells / μL) x Dilution(8)

For analysis and to determine precision, new variable named variation index 1 and 2 were calculated from dispersion of mean values of duplicate samples (tube 1 and 2 respectively). Variation Index(VI) = Sample Mean – Sample Value x 100 Sample Value

Variation measures fluctuation of data. If variation is less or near to zero, the data is consistent enough. If the variation exceeds ten, it indicates the value of CD34+ cells falls outside 10% of the mean for the duplicate samples; the assay needs to be repeated.

For precision, the difference in mean variation was separately observed for 3 groups (Low: <500 CD34+ cells/μL; Medium: 500 to 1000 CD34+ cells/μL and High >1000 CD34+ cells/μL) and the difference in variation 1 and 2 were compared through ‘t’ test.

Statistical Analysis
Continuous variables such as baseline CBC and CD34+ count were summarized in terms of either mean ± SD. The difference in variation index 1 and 2 were compared through ‘t’ test. A P-value of <0.05 was considered to be statistically significant. All the analysis was done using SPSS version 18 (SPSS Inc, Ill, Chicago, USA).

Results
During the study period, CD 34+ cells enumeration was performed for 85 patients (70 autologous and 15 allogenic). The demographic characteristics of autologous patients and allogenic donor are listed in Table 2.

Table 2: Demographic Characteristics of Autologous patients and Allogenic donor sample from Leukapheresis product

<table>
<thead>
<tr>
<th>Sample Value x 100</th>
<th>Autologous</th>
<th>Allogenic</th>
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<tbody>
<tr>
<td>Product Volume (mL)</td>
<td>253±87</td>
<td>247 ±114</td>
</tr>
<tr>
<td>Absolute CD34 (cells/μL)</td>
<td>1119±920</td>
<td>1447±923</td>
</tr>
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</table>

A total of 111 samples collected from PBSC product were analyzed for CD34+ cells enumeration. The absolute count for CD34+ cells were calculated as per ISHAGE protocol (Figure 1). The mean CD34+ count among the samples in the leukapheresis product was 1159 ± 922 cells/μL. The distribution of samples with respect to CD34+ cells concentration was 50% in group high (>1000 CD34+cells/μL) and 25% each in low and medium group.

Reproducibility of samples was tested in duplicate and results were expressed in variation index. We observed no significant difference for mean variation value obtained from sample 1 and 2 separated for Low; Medium and High groups (Table 3). Medium group had lower variation index between the samples than the other groups. The variation index was for all groups were comparable.

Error analysis
Assay was repeated in (10% instances, as the variation index was more than 10. The failed runs were seen with low group (CD34+ count <500 cells/μL) with variation index was more than 10. On repeat runs, the variation index was consistent enough. The failed runs were excluded from results for analysis. In the present study, the difference between the first and second sample measurement seems to be influenced only with CD34+cells concentration <500 cells/μL and the difference is not statistically significant. 

Discussion

Flow cytometric enumeration of CD34+ cells had become widely accepted as the technique of choice to quantify HSCs for the clinical management of stem cell transplantation.(4,8) Several modifications were adopted to simplify the procedure for CD 34+ cells enumeration procedure. The widely accepted and followed guideline presently is ISHAGE.(9) Conventionally, co-efficient of variation (CV) results was used to express precision by replicate measurements. The high CV value suggests more variation between the replicate samples.(4) Gajwoksa et al compared three different methods of CD34+ cell enumeration: the Milan/Mulhouse protocol, the two-platform ISHAGE protocol and single-platform ISHAGE protocol with the aim of verifying the differences between them using CV. Most of the protocol had low CV for all concentrations tested with exception of Milan/Mulhouse assay for low concentration (<50 CD34+ cells/µl). The study concluded single-platform ISHAGE protocol showed high correlation and undoubtedly a more sensitive and reproducible method in CD34+ cells enumeration for mobilized peripheral blood.(4) In the present study we calculated variation Index between the duplicate samples from leukapheresis product. We observed no significant difference in variation index between sample 1 and 2 separated for Low; Medium and High groups (Table 3). Poor reproducibility of CD34+ cells count was observed in low groups (CD34<500 cells/µl) in 10% runs. It is important to note the observed differences in duplicate and understand technical factors for quantitative analysis of CD34 count. The major preanalytical variables that influence enumeration are choice of monoclonal antibodies with fluorochromes, lysing reagent and method, use of microbeads, type of samples like fresh or cryopreserved and volume of sample added. It is well established that centres using lyse no wash or no lyse, no wash method during sample preparation had reduced variability and tighter CV.(10) Similarly accurately measured volume of sample should be pipetted into tube and same accurately measured counting beads to be added.(5) We observed that with low groups (CD34<500 cells/µl), even minute difference in sample volume pipetted might contribute to high variation index or CV.

Table 3: Variation Index for Sample1 and Sample 2 with respect to CD34+ cell concentration in three groups

<table>
<thead>
<tr>
<th>Precision</th>
<th>N</th>
<th>Mean</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low (&lt;500 CD34+ cells/µl)</td>
<td>28</td>
<td>4.2±2.2</td>
<td>0.632</td>
</tr>
<tr>
<td>Medium (500-1000CD34+ cells/µl)</td>
<td>28</td>
<td>2.9±2.3</td>
<td>0.085</td>
</tr>
<tr>
<td>High (&gt;1000 CD34+ cells/µl)</td>
<td>55</td>
<td>3.1±2.2</td>
<td>0.864</td>
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Highly trained operators are required for interpretation of the flow cytometry data. Herbert et al to minimize post analytical errors compared automated analysis of CD34 enumeration flow cytometric data using a probability state model (PSM) with manual ISHAGE gating analysis. The study observed PSM protocol provides higher reproducibility, objectivity and speed with accuracy at least equivalent to ISHAGE model. (11)

In an attempt to address standardization, few international external quality assurance (EQAS) program was established. The overall interlaboratory CV comparison for single platform users was 10%, when compared to 24% in dual platform users. (10) However such programs are not widely popular in
India. Multicentre EQAS program in India might shed some lights on unaccounted variables in our practice. We conclude by testing the CD34 enumeration in duplicate and verifying its repeatability using variation index of less than 10 will help in accurately determining CD34+ graft adequacy in transplant setting.

References: