Comparison Between Total Leukocyte Cell Count From Flowcytometer and Corrected Total Nucleated Cell Count from A Dual Platform Analysis

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ABSTRACT

Umbilical cord blood is an alternate for bone marrow in stem cell transplantation. The treatment efficacy depends on total nucleated cell count (TNCC) and hematopoietic stem cells (HSC) count in the cord blood. Hence nucleated red blood cells (NRBC) enumeration, for quantifying corrected TNCC (cTNCC) becomes important for accurate prediction of clinical transplantation outcome. The objective of this study is to confirm that the total leukocyte cell count (TLCC) is equivalent to cTNCC.
Methods: 20 cord blood samples are used to validate cTNCC obtained from dual platform analysis is equivalent to TLCC obtained using Flowcytometer. TNCC was obtained from hematology analyzer, whereas NRBC and TLCC from flowcytometer.
Results: Statistical significance identified using Passing and Bablok fit showed a very high level of agreement between cTNCC and TLCC
Conclusion: TLCC from flowcytometer can be safely used to report nucleated cell count of cord blood units instead of cTNCC.
Abbreviations: UCB – Umbilical cord blood; HSC – Hematopoitic Stem cells; PB – Peripheral blood; HLA –Human Leukocyte antigen; TNC – Total Nucleated Cells; TLC – Total Leukocyte cells; NRBC – Nucleated Red Blood Cells; TNCC – Total Nucleated Cell Count; TLCC – Total Leukocyte Cell Count; cTNCC – Corrected Total Nucleated Cell Count; ISHAGE – International Society for Hematotherapy and Graft Engineering.

1. INTRODUCTION

Stem cell transplantation is one of the most sought after therapies in case of lethal malignancies and genetic disorders. Umbilical Cord Blood (UCB) is identified as a rich source of Hematopoietic Stem Cells (HSC) and used as an alternate for bone marrow in transplantation. UCB transplantations are carried out to treat patients with haematological malignancies, genetic diseases, immune deficiencies and inherited metabolic disorders (Rubinstein P, 2006). Since the first UCB transplantation to treat Fanconi anaemia in 1988, more than 10,000 transplantations have been carried out to treat different diseases and genetic disorders (Gluckman E et al., 1989).

There are a lot of advantages of using UCB over bone marrow or Peripheral Blood (PB). Some of them are, the immediate availability of the cells, lower risk for donor, lower risk of graft versus host disease, less stringent requirement of Human Leukocyte Antigen (HLA) compatibility between the donor and recipient and comparatively reduced risk of infectious disease transmission (Eapen M et al., 2007).

The dosage volume for transplantation is calculated using absolute TNCC and HSC count. Nucleated Red Blood Cells (NRBCs) are a subset of cord blood cell population. Often, these cells are falsely interpreted and reported as TNCC (Migliaccio AR et al, 2000; Laughlin MJ et al., 2001). Corrected Total Nucleated Cell Count (cTNCC) is a measure of total nucleated cell count without NRBC.

In this study, we compared cTNCC derived from dual platform analysis with TLCC enumerated from flowcytometer.

2. MATERIALS AND METHODS

Umbilical Cord blood (n=20) was collected in a blood bag containing the anticoagulant Citrate Phosphate Dextrose (CPD). Buffy coat of each blood sample was separated by differential centrifugation and sedimentation method. TLCC and NRBC were calculated using the aliquots of the buffy coat in BD FACSCalibur™ (BD Biosciences). TNCC was enumerated using the **Beckman Coulter AcT Diff II Hematology Analyzer**. All the samples were collected and processed within 48hrs. The processed samples were analyzed by BD FACSCalibur™ and BC AcT diff II within 3 to 4 hrs. All the assays were performed based on the standard operating procedures of the stem cell blood bank, LifeCell International Private Limited.

2.1. TNCC Enumeration

TNCC was enumerated using hematology analyzer BC AcT diff 2. This is an electrical impedance based three-part instrument that counts NRBC as a nucleated cell and reports it along with TNCC as white blood cells (WBC).

2.2. TLCC Enumeration

BD FACSCalibur was used to enumerate TLCC. All the reagents used in this assay were ready to use and purchased from BD Biosciences. TLCC assay was performed based on ISHAGE guidelines (Sutherland DR, Anderson L, Keeney M, Nayar R & Chin-Yee I, 1996; Gratama JW, Orfao A, Barnett D, Brando B, Huber A et al., 1988) and TLCC was calculated by using gating (Fig 1, Plot 1 on page no. 2). The samples were diluted to obtain <50,000 nucleated cells/µl using Phosphate Buffer Saline (PBS). To 10µl of FITC-CD45 monoclonal antibodies and 10µl of PE-CD34 monoclonal antibodies, 50µl of well-mixed sample was added to stain leukocytes and HSC. After gentle mixing, the sample was stored in dark for 15 minutes. 1ml of 1x lysing buffer (ammonium chloride based) was added with 10µl of 7AAD followed by gentle mixing. The sample was stored in dark for 15 minutes. 50µl of liquid counting beads was added to the sample and gently mixed.

The instrument was aligned and calibrated using three-color calibrate beads by FACSComp Version 6.0 software (BD Biosciences). The samples were analyzed using CellQuest Pro Version 6.0 software (BD Biosciences).

2.3. Gating

The gating strategy described by Keeney.M et al., (1998) was used to gate the desired population. Threshold was set in FL1 channel based on CD45+ expression such as to exclude debris and include dim CD45+ cells. Since this exclusion channel is

based on CD45+ expression, debris and NRBC's are excluded since NRBCs are CD45- cells. 75,000 total events were collected using CellQuest Pro 6.0 (BD Biosciences) after gentle mixing of the sample. TLCC was enumerated from the exclusion gate where the primary threshold parameter was set (Fig 1, Plot 1 in the same page) To enumerate live TLCC the plot 3 (Fig 1, Plot 3 in the same page) was used where the 7ADD negative events were gated as live TLCC. Counting beads were gated in as in plot 9 (Fig 1, Plot 9 in the same page) since they exhibit fluorescence in both FL1 and FL2 channels.

TLCC per microlitre was calculated using the formula,
TLCC= (No. of TLCC events gated/No. of beads gated) * Concentration of the beads * Dilution factor

Figure 1: Gating strategy used to enumerate CD34+ and CD45+ cells.

Plot 1: The primary threshold channel was set in FL1 to exclude debris and NRBC. TLCC was derived from this gate.

Plot 2: Live TLCC was enumerated from this gate.

Plot 8 : Counting beads were isolated.
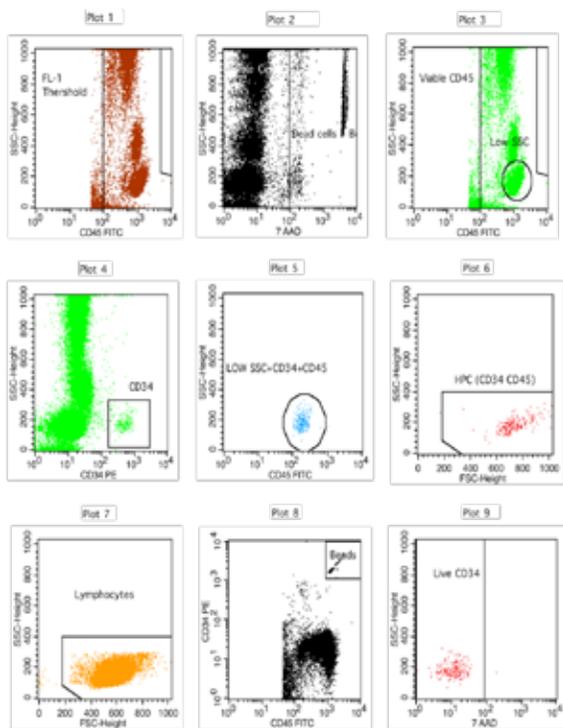
2.4. NRBC Enumeration
NRBCs were enumerated using the methods described by Tsuji.T et al., (1999) and Larghero J. et al., (2006). All the reagents used in this study were ready to use and purchased from Beckman Coulter, Inc. The cells were stained with FITC-CD45 monoclonal antibodies and incubated in dark for 15 min after gentle mixing. PI with hypotonic acid lysing reagent (solution A) was added to the cells and gently mixed. After 3 seconds, hypertonic alkali reagent (solution B) was added. The solution was gently mixed by inverting the falcon tubes for 10 seconds. Samples were analyzed using Cellquest Pro 6.0 (BD Biosciences) in BD FACSCalibur. Using CellQuest Pro 6.0 (BD Biosciences), four different populations were categorized. These were, CD45[+/]PI+ (Leukocytes stained with PI); CD45+/PI- (Leukocytes not stained with PI); CD45-/PI+ (NRBCs) and CD45-/PI- (debris, RBC ghosts and reticulocytes).

NRBCs were expressed as,

NRBC % = (No. of NRBC events/ Total no. of CD45 positive events) * 100

2.5. Correlation
The cTNCC calculated from dual platform is plotted against TLCC. The correlation is checked using Passing and Bablok agreement using 'Analyze it' add on software in Microsoft Excel 2007.

3. RESULTS
A total of 20 samples were used to enumerate cTNCC and TLCC. cTNCC was calculated using TNCC obtained from **Beckman Coulter AcT Diff II Hematology Analyzer** and NRBC% obtained from flowcytometer. TLCC was derived from the CD45+ events from flowcytometer. Results are shown below in a table (Table I, page .

Total number of NRBC's in the sample was calculated as follows,

Total No. TNCC (BC AcT diff II) * of NRBCs NRBC%(BD FACS-Calibur)

Corrected TNCC is calculated using the formula,

cTNCC = TNCC (Beckman Coulter AcT diff II) – Total no. of NRBCs

Table I: TNCC, TLCC and cTNCC values.

S.No	TNCC (cells/μL) Cell counter	NRBC % (Flowcytometry)	Absolute NRBC calculated = TNCC x NRBC%	Corrected TNCC = TNCC- Absolute NRBC calculated	CD45 (cells/μL) Flowcytometry
1	51000	5.4%	2774	48226	44181
2	17400	4.1%	712	16688	16594
3	30700	3.0%	906	29794	26751
4	9600	0.2%	19	9581	7724
5	53900	1.0%	544	53356	41762
6	27500	5.9%	1634	25867	20318
7	64000	5.3%	3386	60614	59747
8	27200	0.6%	163	27037	23401
9	15700	4.3%	681	15019	15097
10	62500	0.2%	100	62400	65357
11	45800	6.4%	2936	42864	34943
12	12900	11.0%	1416	11484	11308
13	39200	10.6%	4136	35064	31054
14	32000	4.8%	1539	30461	26718
15	38700	11.8%	4582	34118	28710
16	26400	10.2%	2693	23707	25000
17	14100	8.9%	1248	12852	11907
18	30600	6.9%	2102	28498	29001
19	72000	25.3%	18216	53784	67239
20	14800	11.5%	1705	13095	11600

3.1. Statistical method used for the analysis
We used Passing and Bablok fit to check the correlation between the cell counts from two different methods. The Passing and Bablok fit is used to see the fit of the model and the Intra-

class correlation (ICC) is used to assess the agreement between the two methods. If the ICC is greater than .80 it's been claimed, as both the methods are similar to each other.

The Passing and Bablok fit (Figure 2) and the ICC of 0.95 (Table II) indicates that cTNCC and TLCC have a very high level of agreement i.e. they are almost exactly the same.

Table II : Intraclass correlation for cTNCC and TLCC

	Intraclass correlation [a]	95% Confidence Interval
Single measures [b]	0.9563	0.8915 to 0.9826

a The degree of absolute agreement among measurements.
b Estimates the reliability of single ratings.

Figure II : Passing and Bablok fit for comparing cTNCC and TLCC.
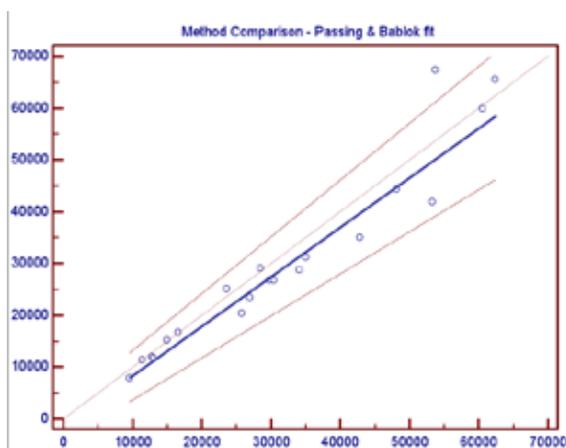
4. DISCUSSIONS

NRBC is present in both newborns and adults. In adults, it is present in very miniscule numbers during normal conditions and can be seen in significant numbers only during times of stressed or abnormal erythropoiesis. On the contrary NRBC are present in much higher concentrations in umbilical cord blood (ranging from 3-10% of WBC concentration). As NRBC are indistinguishable from WBC, they are counted by standard electronic cell counters as WBC (Smogorzewska EM, Dukes L, Kuo L & Kapoor N, 2007). This can result in falsely high TNCC thereby significantly compromising the dosage level available for transplantation (Hanion-Lundberg KM, Kirby RS, Gandhi S & Broekhuizen FF, 1997).

Using a dual platform to obtain cTNCC will possibly limit the potential of the technique in terms of accuracy and precision. Other more accurate method is to calculate TLCC from flowcytometer using ISHAGE guidelines. NRBC can be excluded and only nucleated cells can be counted using flowcytometer (Axt R, Ertan K, Hendrik J, Wrobel M, Mink D & Schmidt W, 1999). It is less labour intensive and far more reliable in terms of accuracy and precision compared to cTNCC obtained from dual platform quantification (Brocklebank AM & Sparrow RL, 2001).

However, it has been reported that the engraftment potential was not affected due to the presence of NRBC in cord blood during transplantation. NRBC is calculated only to report cTNCC and it does not have any clinical significance during cord blood transplantation (Stevens CE et al., 2002). International accreditation bodies like College of American Pathology (CAP) and American Association of Blood Banks (AABB) suggests the stem cell banks to report cTNCC instead of TNCC.

In this study, we established a significant correlation between cTNCC and TLCC. Hence, TLCC can be safely used to report nucleated cell count of cord blood units, obviating the need to perform NRBC enumeration separately.

REFERENCE

1. Rubinstein, P. (2006). Why cord blood? Human Immunology, 67(6), 398-404. | doi: 10.1016/j.humimm.2006.13.015 | 2. Gluckman, E., Broxmeyer, HA., Auerbach, AD., Friedman, HS., Douglas, GW., Devergie, A., ESperou, H., Thierry, D., Socie, G., Lehn, P., et al. (1989). Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. The New England Journal of Medicine. 321(17), 1174-1178. | doi: 10.3205/ctt-2010-en-000079.01 | 3. Eapen, M., Rubinstein, P., Zhang, MJ., Stevens, C., Kurtzberg, J., Scaradavou, A., Loberiza, FR., Champlin, RE., Klein, JP., Horowitz, MM., & Wagner, JE. (2007). Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. Lancet. 369(9577), 1947-1954. | doi: 10.1016/S0140-6736(07)60915-5 | 4. Migliaccio, AR., Adamson, JW., Stevens, CE., Dobrila, NL., Carrier, CM., & Rubinstein, P. (2000). Cell dose and speed of engraftment in placental/umbilical cord blood transplantation: graft progenitor cell content is a better predictor than nucleated cell quantity. Blood. 96(8), 2717-2722. | http://bloodjournal.hematologylibrary.org/content/96/8/2717.long | 5. Laughlin, MJ., Barker, J., Bambach, B., Koc, ON., Rizzieri, DA., Wagner, JE., Gerson, SL., Lazarus, HM., Cairo, M., Stevens, CE., Rubinstein, P., & Kurtzberg, J. (2001). Hematopoietic engraftment and survival in adult recipients of umbilical- cord blood from unrelated donors. The New England Journal of Medicine. 344(24), 1815-1822. | doi: 10.1056/NEJM200106143442402 | 6. Gratama, JW., Orfao, A., Barnett, D., Brando, B., Huber, A., Janossy, G., Johnsen, HE., Keeney, M., Marti, GE., Preijers, F., Rothe, G., Serke, S., Sutherland, DR., Van der Schoot, CE., Schmitz, G., Papa, S. (1998). Flow cytometric enumeration of CD34+ hematopoietic stem and progenitor cells. Cytometry. 34(3), 128-142. | doi: 10.1002/(SICI)1097-0320(19980615)34:3<128::AID-CYTO3>3.0.CO;2-D | 7. Sutherland, DR., Anderson, L., Keeney, M., Nayar, R., & Chin-Yee, I. (1996). The ISHAGE guidelines for CD34+ cell determination by flow cytometry. Journal of Hematotherapy. 5(3) 213-226. | doi: 10.1089/scd.1.1996.5.213. | 8. Keeney, M., Chin-Yee, I., Weir, K., Popma, J., Nayar, R., & Sutherland, DR. (1998). Single platform flowcytometric absolute CD34 cell counts based on the ISHAGE guidelines. Cytometry. 34(2), 61–70. | doi: 10.1002/(SICI)1097-0320(19980415)34:2<61::AID-CYTO1>3.0.CO;2-F | 9. Tsuji, T., Sakata, T., Hamaguchi, Y., Wang, Fs., & Houwen, B. (1999). New rapid flowcytometric method for the enumeration of nucleated red blood cells. Cytometry. 37(4), 291-301. | doi: 10.1002/(SICI)1097-0320(19991201)37:4<291::AID-CYTO6>3.0.CO;2-M | 10. Larghero, J., Rea, D., Brossard, Y., Nifterik, JV., Delasse, V., Robert, I., Biscay, N., Chantre, E., Raffoux, E., Socie, G., Gluckman, E., Benbunan, M., & Marolleau, JP. (2006). Prospective flowcytometric evaluation of nucleated red blood cells in cord blood units and relationship with nucleated and CD34+ cell quantification. Transplantation and cellular engineering. 46(3), 403-406. | doi: 10.1111/j.1537-2995.2006.00736.x. | 11. Smogorzewska, EM., Dukes, L., Kuo, L., & Kapoor N. Nucleated red blood cells in normal bone marrow for transplantation. (2007). Med Wieku Rozwoj. 11(1), 7-11. | | 12. Hanion-Lundberg, KM., Kirby, RS., Gandhi, S., & Broekhuizen, FF. (1997). Nucleated red blood cells in cord blood of singleton term neonates. American Journal Of Obstetrics And Gynecology. 176(6), 1149-1156. | doi: 10.1016/S0002-9378(97)70328-4 | 13. Axt, R., Ertan, K., Hendrik, J., Wrobel, M., Mink, D., & Schmidt, W. (1999). Nucleated red blood cells in cord blood of singleton term and post-term neonates. Journal Of Perinatal Medicine. 27(5), 376-381. | doi: 10.1515/JPM.1999.051 | 14. Wang, FS., Itose, Y., Tsuji, T., Hamaguchi, Y., Hirai, K., & Sakata, T. (2003). Development and clinical application of nucleated red blood cell counting and staging on the automated haematologyanalyser XE-2100. Clinical and Laboratory Haematology. 25(1), 17-23. | doi: 10.1046/j.1365-2257.2003.00476.x | 15. Brocklebank, AM., & Sparrow, RL. (2001). Enumeration of CD34 cells in cord blood: a variation on a single-platform flowcytometric method based on the ISHAGE gating strategy. Cytometry. 46(4), 254-261. | doi: 10.1002/cyto.1136 | 16. Stevens, CE., Gladstone, J., Taylor, PE., Scaradavou, A., Migliaccia, AR., Visser, J., Dobrila, NL., Carrier, C., Cabbad, M., Wernet, P., Kurtzberg, J., & Rubinstein, P. (2002). Placental/umbilical cord blood for unrelated-donor bone marrow reconstitution:relevance of nucleated red blood cells. Blood. 100(7), 2662-2664. | doi: 10.1182/blood.V100.7.2662 |