Functional assessment of cord blood units using two different assays

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Abstract

Background: The cord blood banks and cord blood transplantation community are on the constant lookout for a single objective dependable test that will indicate the functional capabilities of the cord blood units. This should ideally reflect engraftment, proliferation and differentiation capabilities. Presently in vitro Colony forming unit (CFU) assay is being performed by most cord blood centers to determine the functional efficacy of cord blood units. However, technical challenges associated with this assay have made it difficult to standardize the methodology among testing laboratories. A subjective test is associated with variability and non-uniform reporting.

The aim of this study was to evaluate the usefulness of the newly introduced HALO® assay by Hemogenix® by comparing it with the information provided by the traditional colony forming assay (CFU). Repeatability and objectivity were also studied.

Methods: Sixteen Cord Blood units processed in the repository were tested by both, the traditional CFU assay and the HALO® assay.

Results: Our study shows that the CFU assay provides information relating to proliferation and differentiation potential of CBUs while the HALO® assay gives quantitative output with reference to proliferative capability of CBUs.

Conclusion: While both the assays provide valuable information on the functional efficacy of cord blood units from different stand points, one test perhaps cannot be substituted for the other. It is good to have both the tests available to bring in some additional information on its capabilities, which is much better than providing only one value with high subjectivity component.

Keywords: Cord blood units, Colony forming assay, HALO assay, Potency assay, Functional efficacy evaluation.

Introduction

Umbilical Cord Blood (UCB) has emerged as a feasible alternative source of hematopoietic progenitors for allogeneic stem cell transplantation, primarily in patients who lack HLA-matched marrow donors (1-6). Since the first case of successful UCB transplantation, there have been more than 25,000 cord blood transplants performed for a variety of malignant and non-malignant diseases (7-8).

The present challenge of Cord blood banks is to provide quality products for successful transplantation. In all fairness, it should be emphasized that until recently, Colony Forming Unit (CFU) assay was the only assay available that could provide some indication that the cells present in the processed product would proliferate and differentiate. Even though, proliferation and differentiation are intricately intertwined from a biological point of view, from an assay point of view both of these are farther apart. Among the various parameters tested, HLA reports, total nucleated cell count (TNC), absolute nucleated cell count, cell viability, CD34$^+$ cell content and colony forming units (CFUs) of the cord blood samples are identified as the determinant of quality & functional potential. The traditional 14 day

CFU assay is the only available in-vitro colony forming assay that determines the functional ability of the graft (9). It is believed that colony forming cells (CFCs) are directly responsible for engraftment and long term reconstitution of the hematopoiesis in vivo, post transplantation. This assay, even though, first published in 1966, was never designed as a cell potency assay (10). It suffers with numerous problems and deficiencies. It is time consuming to perform, requires manual enumeration of colonies & technical expertise, and moreover cannot be calibrated and standardized as no external standards have been established. With a number of regulatory bodies setting up guidelines for cell and tissue processing for clinical use, it is even more relevant than ever before. AABB and FACT guidelines insist on carrying out functional potency assay for determining the quality of the cord blood units before clinical use.

The HALO® assay established and marketed by Hemogenix® is one of such an assay which has been developed as a potency assay. It is a suspension expansion assay that directly measures proliferative potential of cells after 5 days of incubation. During incubation, the cells stimulated by growth factor combinations, proliferate & divide. Cluster of cells begin to form in the semi solid medium due to immobilization. If not left to mature into colonies, these clusters contain proliferating cells capable of producing ATP (Adenosine tri phosphate). At a time when proliferation is increasing, the cells in the culture are lysed to release iATP (intracellular ATP) in the surrounding medium. iATP acts as a limiting substrate for the following reaction:

luciferase

$$iATP + luciferin + O_2 \longrightarrow Oxylucyferin + AMP + PPi + CO_2 + Bioluminescence\ light.$$
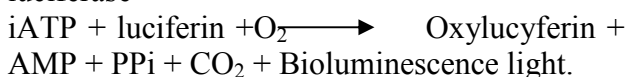
The released ATP drives the luciferase reaction to produce bioluminescence in the form of light which can be detected & measured in a plate luminometer using a 96 well plate.

Traditional CFU assay focus on the proliferation and differentiation potential of the cells, while the HALO® assay essentially is based on the proliferation potential. While, the CFU assay thus helps us in determining the type of cells that can be formed from the available stem cells, HALO® assay indicates only the functionality due to the ATP released by the proliferating cells.

Materials and Methods
Cell Source
Cord blood banking is a licensed activity in India. The public and the private banking at Reliance Life Sciences Pvt Ltd. are accredited by the AABB.
Sixteen Cord blood units (CBU) obtained from mothers after a due consenting process were used for the study. All necessary ethics committee approvals for this activity are in place.

Processing of umbilical cord blood
All the processes were carried out in cGMP compliant facility. Pre-process aliquots were taken for whole blood count (0.4 ml) and blood grouping (1ml).The cord blood samples were depleted for RBCs followed by centrifugation and excess plasma removal to obtain a leukocyte rich fraction. Post-process aliquots from this leukocyte rich fraction were taken for CFU assay (0.3ml), cell count and CD34 + cell enumeration (0.7ml). An aliquot for retention was (1ml) stored in -80°C. The remaining leukocyte rich fraction was then cryopreserved under controlled rate using standardized & validated procedures.

Immunophenotyping
The post-process aliquot, after taking the cell count, was taken for characterization of surface markers using flow cytometer (FACS Calibur, Becton Dickinson (BD), USA) following the ISHAGE protocol. Briefly, the cells were labeled with CD34 (BD Pharmingen, San Jose) and CD45 (Immunotech, Beckman Coulter, CA) antibodies against their respective Isotype Controls (BD Pharmingen, San Jose) and were incubated at 4^0C followed by depletion of RBCs using the lysis buffer . To obtain percentage of viable cells, the cells were incubated with Viaprobe (BD Pharmingen, San Jose) before acquisition. The results thus obtained from flow cytometer were in terms of percent viable CD34^{+} cells. The analysis of the data was done using CellQuest software on BD FACS Calibur.

Colony forming assay

The cells from the post-process aliquot, prior to cryopreservation were cultured in methyl cellulose based semi-solid media containing recombinant IL-3, stem cell factor, erythropoietin and GM-CSF. 100, 000 cells were taken for the assay. The required numbers of cells were then diluted 1:5 using dextran as the diluent and mixed with 3.0 ml of methyl cellulose (Stem cell technologies, Canada). 1.1ml of this cell suspension was then plated in duplicates. The plates were then incubated in humidified chambers at 37^0C and 5% CO_2 for 14 days. The type and the number of colonies generated after 14 days were enumerated using the phase contrast microscope. The colonies were evaluated morphologically. A colony was defined as clusters consisting of ≥40 cells. The average of the two plates was considered for calculating the total number of colonies/CBU. The test was done by two individuals independently.

HALO assay

The HALO assay was also performed on the same set of samples by carefully following the manufacturer's instructions (Hemogenix, USA). Briefly, the frozen Master mix tubes containing 0.9 ml cell culture medium were transferred to the 37^oC incubator. The contents of each tube were thoroughly mixed using a Vortex mixer and 50,000 cells were added to the master mix tube followed by dispensing 100µl of Culture Master mix per well in six replicates in the 96 well plate, so that the final number of cells in a well is 5000. The culture plates were incubated for 5 days in humidified chambers at 37^0C and 5% CO_2. Just prior to sample luminescence measurements, an ATP dose response was performed to ensure that the reagents and luminometer are working correctly. The dilutions of ATP standards were prepared for ATP dose response and 100µl of each was added into the corresponding wells. 100 µl from the low and high control vials were also transferred to the designated wells. The standards & controls were then treated with ATP lysing / monitoring reagent to release the ATP into the surrounding environment. After 2 mins of incubation the bioluminescence was measured in a plate luminometer (Model-Optima Polstar, BMG Labtech).

Results

Immunophenotype of Hematopoietic Stem cells (HSCs)

The immunophenotypic analysis of all the cord blood samples was done following the ISHAGE protocol. A representative figure is shown as Fig.1. The possible CD34 values ranged from 0.01% to as high as 14% (Fig.2). These differences were attributed to the possible biological variations within the samples.

Clonogenic potential of HSCs

Following incubation for 14 days, we observed early progenitor colonies like the BFU-E (burst forming units – erythroid) and CFU-GEMM (colony forming units – granulocyte, erythroid, monocyte and macrophage cells), along with few more mature colony forming units like the CFU-E (CFU-erythroid), CFU-GM (CFU-granulocyte, monocyte), CFU-G (CFU-granulocyte) and CFU-M (CFU- monocyte) (Fig.3). The percentage of each of the type of colonies varied from sample to sample but most types of colonies were observed in every sample. The type and the number of colonies were scored using a simple scoring system.

Proliferation potential of HSCs using Hemogenix® assay

The 'HALO® assay principle states that the amount of bioluminescence produced is directly proportional to the proliferation status of the cells. The RLU reading is converted to µM concentration of ATP to determine if the sample falls within acceptable limits (Fig. 4a). As shown in fig 4b, sample 6 of the first sample set showed less than 0.4 µM concentration of ATP, thereby falling below the acceptable limits. If we were to take this as an indicator of functionality then, this sample would be rejected for banking. Sample 5 of first sample set and sample 1 of second sample set are on the borderline and hence such samples have a high probability of rejection. Other samples could be then considered for transplantation purposes, provided all other factors are suitable.

Comparison of the CFUs with the Mean RLUs per CBU

A series of comparative analysis were performed to determine whether any relationship existed between the TNCs, the

absolute CD34^{+} cell content, the CFU and the RLUs of each of these 16 samples (Fig 5). The TNCs and the absolute CD34^{+} cell content were the only two parameters which co-ordinate well with each other as shown in fig 5a. The samples having lower TNCs showed lower CD34^{+} cell content and higher counts had higher CD34^{+} cell content. The absolute CD34^{+} cell content and the CFU per unit also showed a decent co-ordination amongst themselves as shown in fig. 5b. But the CFU and the RLUs did not show any correlation amongst each other which is quite understandable as it is a function of TNC in the sample. (Fig 5 c-d).

Discussion

In this study, we have tried to look for any correlation between the TNCs, the absolute CD34^{+} cell content, the colony forming ability by traditional clonogenic assays and the RLUs by the HALO® assay. We observed that there exists reasonable correlation between the TNCs and the absolute CD34^{+} cell content of each of the cord blood unit and as also between the CD34^{+} cell content and the CFUs. We have not been able to show any satisfactory correlation between the other parameters studied.

The CFC assay measures the number of progenitors capable of producing colonies and the functional ability of these progenitors to produce colonies belonging to specific cell lineages, but does not quantify the proliferation potential (11) The HALO assay measures the proliferation potential, but does not measure the differentiation potential. The differences underlying the principles of both these assays are probably responsible for the variation observed between their readouts. The colony forming assay counts only the numbers of colonies and the large or the small colonies do not reflect the higher or lower proliferation potential of the different cells. This perhaps means that UCB units having low ATP and high CFU values may have large number of progenitors in the cord blood unit that have high clonogenic potential, but low proliferation response, while a high ATP and low CFU values determine a CBU with less number of colony forming units having extremely immature cells that possess very high proliferation potentials. Therefore, it is very difficult to say if one assay will accurately

convey the functional potential of the cord blood unit.

Other reasons are also attributed to the variation observed between the results by the two assays. One of the important reasons seems to be the growth factor cocktail used for these assays as shown by Reems et al. For the CFU assay, we have used methylcellulose that contains IL-3, stem cell factor (SCF), erythropoietin (EPO) and GM-CSF, while the HALO® assay uses EPO, GM-CSF, IL-3, IL-6, SCF, TPO and Flt-3 ligand. TPO and Flt-3 ligand help in proliferation of stem cells and IL-3 supports the proliferating cells during culture. Different cytokine cocktail have different effect on the proliferation and the differentiation of the cord blood stem cells.

While HALO® assay is entirely instrument based that reduces the interpersonal variation , clonogenic assays are entirely manual. If clonogenic assays are automated, the variations could be minimized.

Burger et al have shown that a dramatic reduction in CFC assay variability can be achieved by taking a few precautions such as performing pre- and post dilution TNC counts, use of an automated dilution calculator and plating cells using electronic pipettes (12).

The HALO® assay and the CFC assays are therefore, based on two different platforms and principles and hence, correlation between these two assays is not possible. Both these assays provide some valuable information which are complimentary to each other in defining the potency of each cord blood unit.

This study, therefore, suggests that both the assays may be necessary for quantitatively measuring the biological activity of the cord blood unit as per the requirement of the regulatory bodies governing the cord blood banking and transplantations.

Legends
Figure 1
Representative figure for ISHAGE protocol. CD45 positive cells are first gated on the entire

population of cells to obtain R1. Of the CD45^{+} cells, the CD34^{+} cells are selected from the total CD45^{+} population, R2. R5 is the region where the non-viable cells are gated out from the entire cell population. Thus, when we read the result of R4, we get percentage of viable CD34^{+} cells.
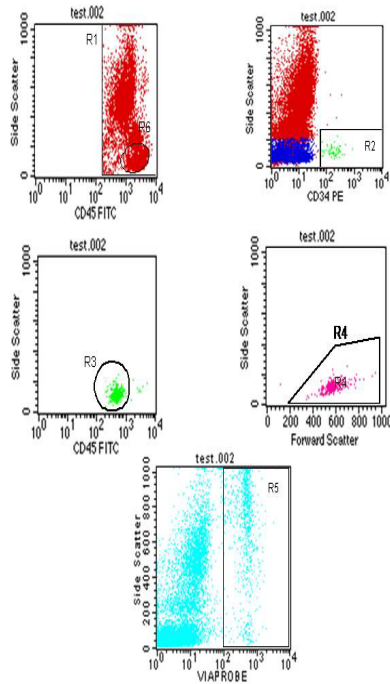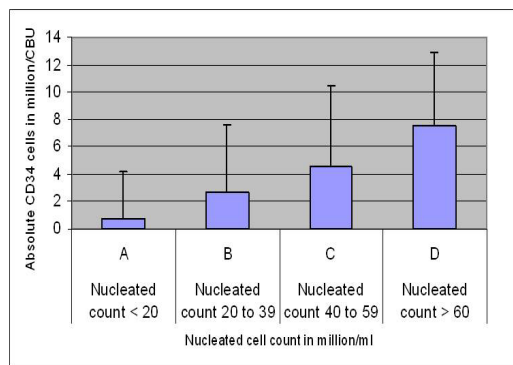
Figure 1

Figure 2

A set of 10 CBUs were analyzed for various ranges of nucleated cell count to see if there exists any correlation with absolute CD34 counts. It was found that there was a direct correlation between the two. The absolute CD34 count generally increases with increase in the total nucleated cell count.

Figure 2

Nucleated cell count v/s Absolute CD34 count

Figure 3

CFU-GEMM (colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte) giving rise to colonies containing multiple lineages of cells usually including erythroid cells.

CFU-GM (Colony forming unit-granulocyte, macrophage): Clonogenic progenitors of granulocytes (CFU-G), macrophages (CFU-M), or both (CFU-GM) containing a minimum of either 20 or 50 of these cells.

BFU-E (burst forming unit-erythroid) a class of more primitive erythroid progenitors than CFU-E. The distinguishing property of the BFU-E is its greater proliferative capacity which enables it to give rise to larger, multi-clustered erythroid colonies than those produced from CFU-E.
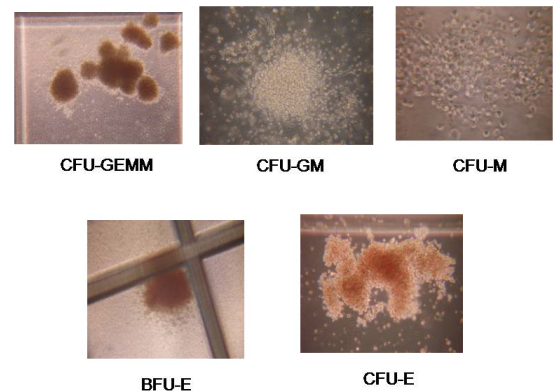
Figure 3

Figure 4

This is a standard curve which is plotted using known values of ATP. This standard curve is used as a reference to obtain the concentrations of the iATP in μM values.

The cut-off ATP concentration for acceptance limits is of 0.04uM. Sample 6 (of set 1) would be rejected. Since, Sample 5 (of set 1) & Sample 1 (of set 2, in red) are on the borderline, would probably also be rejected for use. However rest all samples would be accepted. The high ATP value may indicate the presence of primitive stem cells that may provide long-term engraftment and reconstitution

Figure 5

Comparative analysis of total nucleated cell counts (TNCs), the CD34^{+} cell content, the CFU and the RLUs of each of the cord blood samples. (a) Shows correlation between the

TNC and absolute CD34$^+$ cell count. (b) Shows correlation between the absolute CD34$^+$ cell content and the CFU per CBU. No correlation observed between the (c) RLU and the absolute CD34$^+$ cells and (d) RLU and the CFU of each sample. (e) Shows a comparative bar graph analysis of TNC, absolute CD34$^+$ cell content and the CFU assays.
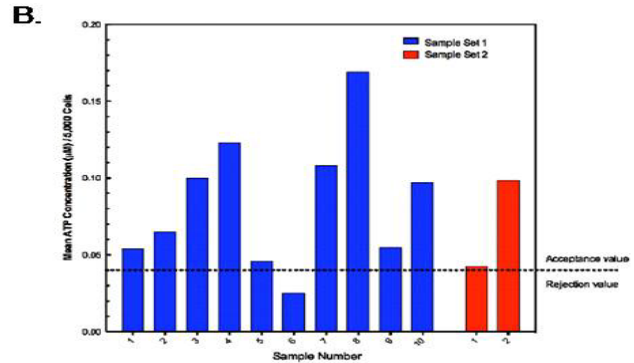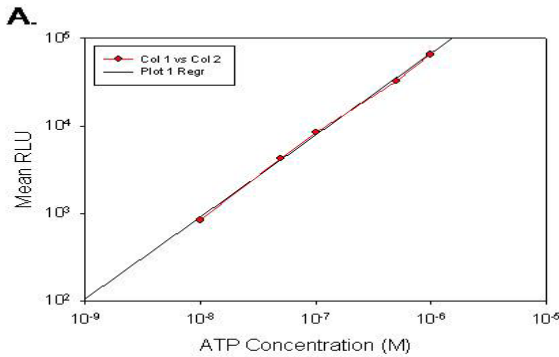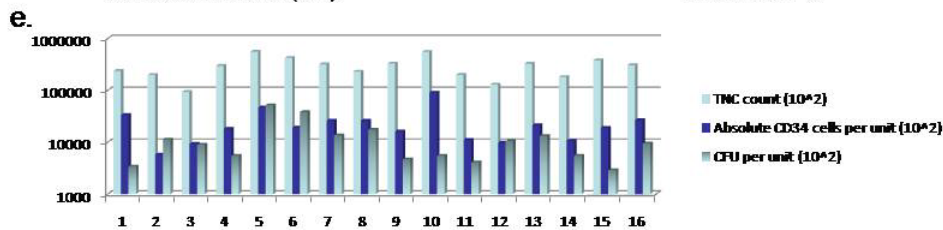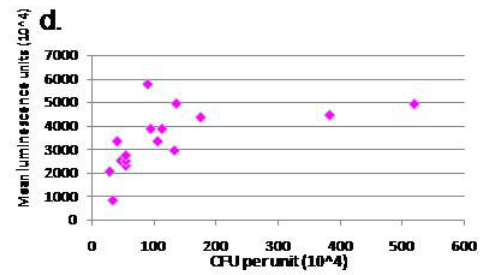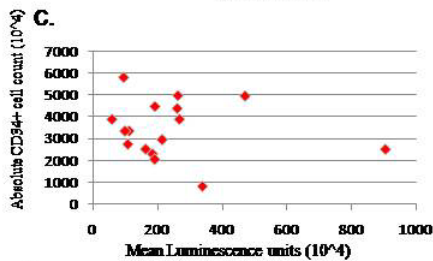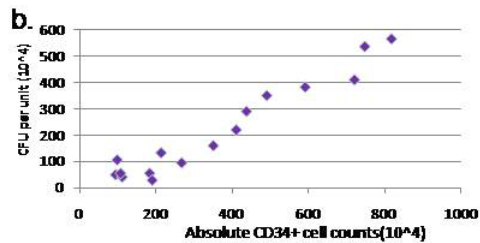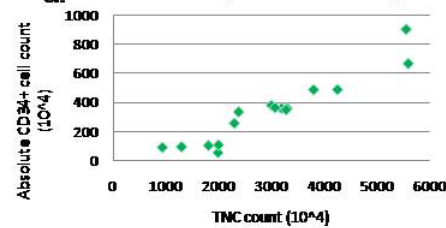
Figure 4

Figure 5

References

1. Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A et al; Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA identical sibling. N Engl J Med 1989; 321:1174-8.
2. Wagner JE, Kernan NA, Steinbuch M, Broxmeyer HE, Gluckman E. Allogeneic sibling umbilical-cord-blood transplantation in children with malignant and nonmalignant disease. Lancet 1995; 346:214-99.
3. Rubinstein P, Carrier C, Scaradavou A, Kurtzberg J, Adamson J, Migliaccio AR et al; Outcomes among 562 recipients of placental-blood transplants from unrelated donors. N Engl J Med 1998; 339:1565-77.
4. Wagner JE, Barker JN, DeFor TE, Baker KS, Blazar BR, Eide C et al; Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment related mortality and survival. Blood 2002; 100:1611-8.

5. Rocha V, Labopin M, Sanz G, Arcese W, Schwerdtfeger R, Bosi A et al; Acute Leukemia Working Party of European Blood and Marrow Transplant Group; Eurocord-Netcord Registry. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. N Engl J Med 2004; 351:2276-85.

6. Martin PL, Carter SL, Kernan NA, Sahdev I, Wall D, Pietryga D et al; Results of the cord blood transplantation study (COBLT): outcomes of unrelated donor umbilical cord blood transplantation in pediatric patients with lysosomal and peroxisomal storage diseases. Biol Blood Marrow Transplant 2006; 12:184-94.

7. Rocha V, Broxmeyer HE. New approaches for improving engraftment after cord blood transplantation. Biol Blood Marrow Transplant. 2010: 16 (1 Suppl): S 126 – 132.

8. Aljitawi O. Ex vivo expansion of umbilical cord blood: where are we? Int J Hematol 2012: *doi 10.1007/s12185-012-1053-6.*

9. Measurement of hematopoietic stem cell potency prior to transplantation. White paper, Hemogenix 2009.

10. Cell Potency assays for the 21st century stem cell transplantation and cord blood storage and processing laboratory. White Paper, Hemogenix, 2007.

11. Jo-Anna Reems, Karen M. Hall, Luladay H. Gebru, Greta Taber, and Ivan N. Rich. Development of a novel assay to evaluate the functional potential of umbilical cord blood progenitors. Transfusion 2008; 48: 620-628.

12. Burger SR, Kadidlo D, McCullough J. Improved progenitor assay standardization using peripheral blood progenitor cells from a donor treated with granulocyte-colony stimulating factor. Transfusion 1999; 39:451-6