THE TOTAL NUCLEATED CELL (TNC) FRACTION AND DYE EXCLUSION VIABILITY CANNOT BE USED TO MEASURE THE QUALITY AND POTENCY OF UMBILICAL CORD BLOOD CELLS IN SEGMENTS AND UNITS

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This study has now been published in the JOURNAL OF TRANSLATIONAL MEDICINE (2015) 13:94 with the title:
"Detecting primitive hematopoietic stem cells in total nucleated and mononuclear cells fractions from umbilical cord blood segments and units"

The "Null Hypothesis" and Methods

Virtually all cord blood unit (CBU) measurements are based on total nucleated cell (TNC) counts, from the decision to store a CBU, to release for transplantation and correlation with clinical outcome (time to engraftment). The "Null Hypothesis" states that measurement of stem cells in the UCB should not be dependent upon the purity of the UCB preparation. In short, there should not be a difference between measuring UCB stem cells in a TNC or mononuclear cell (MNC) fraction.

Essential Methods

- CBUs obtained from two cord blood banks, one using Sepax the other AXP technology.
- Total of 63 frozen individual segments (~0.1mL) and 10 units with segments tested.
- TNC and cell differential of thawed segments and units measured using Medonics.
- MNC count determined by Z2 particle counter after density gradient centrifugation.
- Dye exclusion viability of 7-AAD performed by flow cytometry.
- CFU assay performed using CAMEO™-4 (HemoGenix).
- ATP bioluminescence assay performed using HALO®-96 SPC-QC & PQR (HemoGenix).

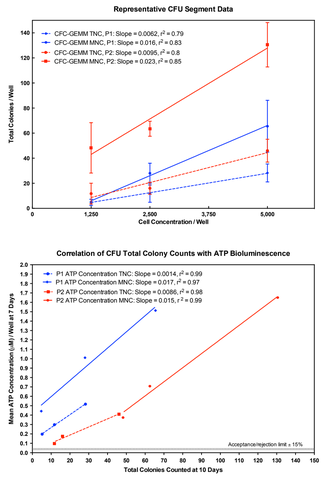
Stem Cell Populations Detected

CFC-GEMM: Primitive hematopoietic stem cell.
HPP-SP: Primitive lympho-hematopoietic stem cell.

Assay Readouts

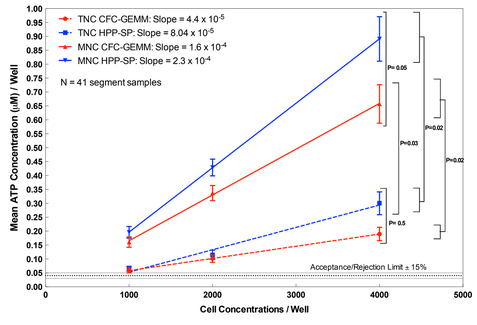
HALO®: ATP concentration at any cell dose = Stem cell proliferation ability or "quality"
HALO®: Slope of ATP dose response = Stem cell proliferation potential or primitiveness.
CFU: Same as HALO, but determines differentiation ability and potential.

1. Verification of the CFU Assay Against HALO



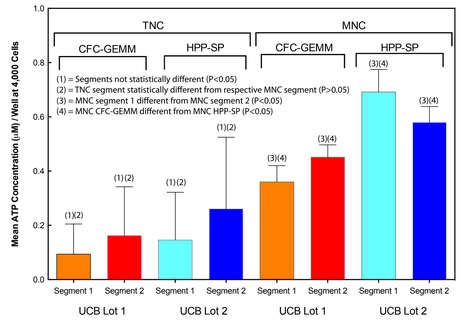
Upper panel: Detection of CFC-GEMM derived from TNC and MNC, performed by 2 different people on the same day from two segments of the same UCB lot.
Lower panel: In addition to the CFU, an ATP bioluminesence proliferation assay was performed on the same cells .
Conclusions: High correlation between CFU and HALO verifies the assays against each other. TNC produces significanly lower values than MNC fraction, indicating an underestimation of both proliferation and differentiation ability and potential.

2. The TNC Fraction Masks and Severely Underestimates Cord Blood Stem Cell "Quality" and Potency



Results: The steeper the slope, the more primitive the cells. The slopes for TNC and MNC do not match. Measurement of proliferation ability and potential of CBU segments from TNC and MNC fractions for CFC-GEMM and HPP-SP clearly demonstrates that the TNC fraction masks and severely underestimates the functional capacity of the stem cells in the segments. A more advanced form of this analysis is potency shown in Fig. 7.
Conclusions: As expected, the HPP-SP is more primitive than the CFC-GEMM and exhibits a greater proliferation potential. **The TNC fraction cannot be used to measure stem cell "quality" or potency of a UCB unit. The "Null Hypothesis" is rejected.**

3. The TNC Fraction Cannot Distinguish between Stem Cell "Quality" of Segments from the Same UCB Lot



Primitive hematopoietic and lympho-hematopoietic stem cell "quality" was analyzed in two segments from the same cord blood unit in TNC and MNC fractions.
Result: The TNC fractions from each of the two segments were statistically indistinguishable, whereas those from the MNC fraction could be shown to be statistically significant from each other.
Conclusion: The TNC fraction can produce a false interpretation indicating that segments of the same cord blood unit are similar, when in fact the opposite result occurs.

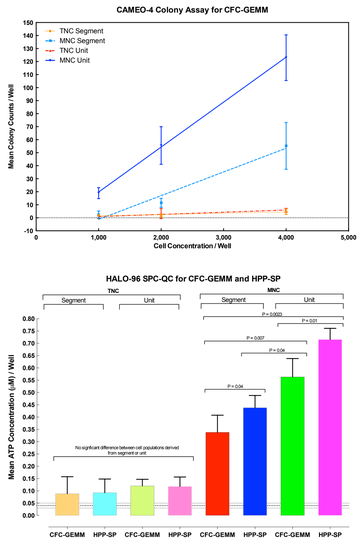
4. Comparison between UCB Samples from 2 Cord Blood Banks

Cell Concentration	Cell Population	Cord Blood Bank 1 (N = 12) TNC (mean± SEM, %CV)	Cord Blood Bank 1 (N = 12) MNC (mean± SEM, %CV)	Cord Blood Bank 2 (N = 29) TNC (mean± SEM, %CV)	Cord Blood Bank 2 (N = 29) MNC (mean± SEM, %CV)
1,000	CFC-GEMM	0.09±0.03, 91.9%	0.238±0.06, 64.5%	0.057±0.01 91.7%	0.122±0.01, 60.6%
	HPP-SP	0.099±0.03, 99.7%	0.323±0.06, 56.5%	0.06±0.01, 90.3%	0.146±0.02, 58.7%
2,000	CFC-GEMM +	0.165±0.04, 74.2%	0.544±0.1, 57.6% [1]	0.085±0.01, 86.3%	0.25±0.02, 46.1% [1]
	HPP-SP *	0.21±0.06, 79.6%	0.723±0.13, 56.2% [2]	0.126±0.03, 111.0%	0.308±0.03, 79.7% [3]
4,000	CFC-GEMM *	0.284±0.08, 87.4%	0.887±0.16, 58.2% [2]	0.177±0.03, 81.9%	0.499±0.05, 49.0% [4]
	HPP-SP *	0.396±0.1, 73.0%	1.352±0.2, 47.4% [2]	0.293±0.05, 87.8%	0.704±0.06, 45.9% [4]

+ represents 2,000 cells/well between MNC from CBB1 vs CBB2, P = 0.05
* represents MNC from CBB1 vs CBB2, P < 0.001
[1] represents TNC vs MNC from CBB1, P = 0.05
[2] represents TNC vs MNC from CBB1, P < 0.001
[3] represents TNC vs MNC from CBB2, P = 0.05
[4] represents TNC vs MNC from CBB2, P < 0.001

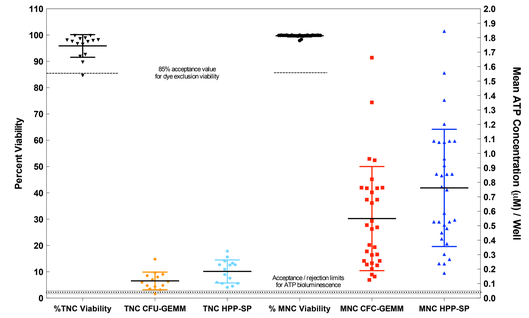
Stem cell "quality" (proliferation ability) and potential were analyzed from multiple segments prepared by two different cord blood banks (CBBs). CBB1 used Sepax, while CBB2 used the AXP processing system.
Results: Although it appeared that CBB1 produced a slightly higher "quality" of both TNC and MNC, the TNC fractions from both CBBs produced equally abysmal results as far as stem cell "quality" and potency were concerned.
Conclusion: Both CBBs could not produce UCB samples that provided an adequate representation of stem cell "quality".

5. Is the Cord Blood Segment Representative of the Unit?



Using both CFU and HALO, the segment and unit were analyzed to determine if the segment was a true representation of the unit.
Results: This shows a single example of 10 segments and units tested. In each case, the TNC fraction from the segment and unit produced similar results, whereas the MNC fraction allowed the segments to be statistically distinguished from the unit.
Conclusion: Using TNC, a false interpretation occurs leading to the conclusion that the segment is representative of the unit. The MNC fraction produces the opposite conclusion.

6. Dye Exclusion Viability Produces a False Positive Indication of Cord Blood Functionality



For every segment and unit tested, viability by 7-AAD was tested before (TNC) and after (MNC) fractionation.
Results: Virtually all TNC samples produced viabilities of >85% after thawing. All MNC viabilities were near 100%. Metabolic viability using ATP bioluminescence of the stem cells did not correlate with 7-AAD.
Conclusion: Dye exclusion viability by 7-AAD produces a false positive result, which is interpreted as indicating that the cells are functional, when in fact they may not sustain proliferation or are metabolically dead.

7. Measurement of Cord Blood Potency

Potency can only be measured using a quantitative and validated assay that measures the biological activity of the "active" components (in this case, stem cells), that are responsible for the intended response (in this case, engraftment). The measure of potency is the potency ratio that can only be estimated using a reference standard (potency = 1) of the same material, i.e. cord blood. 25% of samples have low potency and would be rejected; this corresponds to ~24% graft failure rate (see (11) below).

CONCLUSIONS & CONSEQUENCES of CURRENT TESTING

1. Current tests show little metrology and are inaccurate and insensitive.
2. HALO ATP bioluminescence assay verified against CFU assay.
3. TNC fraction dilutes, masks and severely underestimates the functional capacity of the unit.
4. Current processing to the TNC fraction cannot be used to measure stem cell "quality" and potency. Threshold for storing or discarding CBUs is falsely determined.
5. The "Null Hypothesis" is rejected.
6. Segments do not compare to each other or the unit.
7. Dye exclusion viability produces false positive results leading to a misleading indication of functional ability.
8. Cord blood has to be further purified to MNC fraction.
9. A validated potency assay must be performed at least after cryopreservation and before release to ensure stability and consistency of the stem cells prior to use.
10. Lack of measurement have led to false assumptions and inferences have been made, i.e. assuming the presence, quality and potency of stem cells without measuring them.
11. High graft failure rate (1 in 5 patients) due to low or lack of potency.
12. Not a single unit tested for stem cell "quality" or potency out of >730,000 units collected and >35,000 CB transplants performed worldwide, and >206,000 units collected and >5,000 CB transplants in the U.S.
13. Non-compliance with the U.S. Stem Cell Therapeutic and Research Act.
14. There is a dire need to change current UCB testing practices.