A Rapid and Accurate Method for the Stem Cell Viability Evaluation: The Case of the Thawed Umbilical Cord Blood

Vassilios Katsares, PhD, Anastasia Petsa, PhD, Antigonos Felesakis, B Sc, Zissis Paparidis, PhD, Eleni Nikolaidou, B Sc, Sofia Gargani, B Sc, Iliana Karvounidou, B Sc, Karina-Alina Ardelean, B Sc, Nikolaos Grigoriadis, B Sc, John Grigoriadis, B Sc

(Biogenea-Cellgenea Ltd, Trade Centre "Plateia," Thessaloniki, Greece)

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Abstract

The umbilical cord blood (UCB) is a major source of hematopoietic progenitor cells. These cells can be cryopreserved for possible future use. In order to evaluate the viability of cryopreserved progenitor cells, a small amount of cells is thawed and subjected to the trypan blue exclusion test in order to count the dead (blue) cells and the live (white) cells.

We used the Promega Glo-Max luminometer along with the CytoTox-Glo Cytotoxicity kit and the manufacturer's protocol as an alternative and rapid assay compared to the traditional trypan blue exclusion test.

The calculated viability percentages were similar between the 2 assays, but the counting time for a whole 96-well plate is only 30 min.

The CytoTox-Glo kit is a time-saving assay for the accurate evaluation of progenitor cell viability following cryopreservation and thawing.

After reading this article, readers should understand a rapid method for the cell viability evaluation. This method is as accurate as the trypan blue exclusion test, but one can evaluate simultaneously 96 samples in half an hour.

Blood Banking 60901 questions and corresponding answer form are located after this CE Update on page 561

During the 1970s, it was demonstrated that the umbilical cord blood (UCB) contains hematopoietic progenitor cells. The suggestion that cryopreserved UCB could be used as a source of stem cells in much the same way as transplantation of bone marrow paved the way for the first successful human UCB transplant, performed in France in 1988.[1]

Umbilical cord blood is regarded as a viable source of hematopoietic stem and progenitor cells (HSC/HPC). The UCB transplants provide the advantage of requiring less stringent human leukocyte antigen (HLA) matching between donors and recipients. Furthermore, UCB transplants usually lead to hematopoietic engraftment with lower rates of graft versus host disease (GvHD).[2]

Umbilical cord blood hematopoietic cells have been employed successfully as a therapeutic source of autologous and allogeneic transplants for more than 20 years.[3]

Based on these facts, many cord blood banks (CBBs) have been developed worldwide with the aim of increasing the pool of hematopoietic stem cell donors available for transplants.[4] Consequently, the use of autologous or allogeneic UCB-derived cell transplantation in the treatment of various diseases is expanding, along with an increase in UCB banking.[5] It is suggested that in 1 mL UCB there are approximately 8,000 primitive erythroid progenitors (BFU-E), between 13,000 and 24,000 myeloid progenitors (colony-forming units-granulocyte/macrophage [CFU-GM]), and between 1,000 and 10,000 multipotent progenitors (CFUgranulocyte/erythroid/ macrophage/megakaryocyte [CFUGEMM]).[6]

Cord blood banking involves the following phases: i) recruitment, consent, and testing of maternal donors; ii) collection of the UCB unit; iii) processing, freezing, and testing of the UCB unit; and iv) release of cord blood unit to the transplant center.[7]

The transplantation of UCB cells requires both efficient collection and cryopreservation procedures to obtain an acceptable cell yield and recovery. The purpose of different cryopreservation methods is to minimize cell injury during the freeze/thaw process (cryoinjuries). The cell injury may be the result of extensive cellular dehydration, due to osmotic gradient and/or intracellular ice crystallization.

To prevent cryoinjuries, it is necessary to establish the optimal cooling rate and concentration of cryoprotectant, which represent 2 main factors for the survival of frozen cells. The best cell recovery is after controlled freezing with 5–10% dimethylsulphoxide (DMSO), but a unique, accepted, and standardized cryopreservation protocol has not yet been established.[8]

The viability of thawed hematopoietic progenitor cells is traditionally evaluated with the trypan blue exclusion test. Viable cells exclude trypan blue stain uptake, due to their intact cell membranes; nonviable cells, however, are membrane-porous cells which stain blue. This method requires a technician, a hemocytometer, and a light microscope to enumerate both stained and unstained cells. The percentage viability is then calculated.[9]

Apart from the manual method, there is an automated system for viability evaluation, using a PC and a Charge-Couple Device (CCD) camera connected to a machine that uses trypan blue for the automated enumeration of dead and viable cells.[9]

Recently, a novel luminescent system was developed to evaluate the cytotoxicity of several cytotoxic agents. There are several different kits that can be used with the Glo-Max system including the CytoTox-Glo Cytotoxicity Assay. This is a luminescent cytotoxicity assay that measures the relative number of dead cells in cell populations. The CytoTox-Glo Assay measures the extracellular activity of a distinct intracellular protease (dead-cell protease) when the protease is released from membrane-compromised cells. A luminogenic cell-impermeant peptide substrate (AAF-aminoluciferin) is used to measure dead-cell protease activity. The released aminoluciferin product is measured as "glow type" luminescence generated by Ultra-Glo

Recombinant Luciferase provided in the assay reagent. The AAF-aminoluciferin substrate cannot cross the intact membrane of viable cells and does not generate any appreciable signal from the live-cell population. The amount of luminescence correlates directly with the percentage of cells undergoing cytotoxic stress. With the addition of a lysis reagent, the CytoTox-Glo Assay can also deliver the luminescent signal associated with the total number of cells in each assay well. Viability can be calculated by subtracting the luminescent dead-cell signal from the total luminescent value, thus allowing you to normalize assay data to cell number and mitigate assay interferences that may lead to erroneous conclusions.[10-12]

Glossary

CBB	Cord Blood Bank
CCD	Charge-Coupled Device
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate-Buffered Saline
GVHD	Graft versus Host Disease
HLA	Human Leukocyte Antigen
HPC	Hematopoietic Progenitor Cell
HSC	Hematopoietic Stem Cell
RLU	Relative Luminescence Units
UCB	Umbilical Cord Blood

Materials and Methods

Cell Processing, Freezing, and Thawing

Cord blood units are collected during labor by trained midwives and obstetricians with the placenta in utero immediately after clamping the cord and separating the infant. The collection bags (T2959; Fresenius Kabi AG, Bad Homburg, Germany; anticoagulant citrate phosphate dextrose 20 mL aggregate volume) are placed in a low position to facilitate blood flow by gravity.[13] An informed consent document is signed by all the donors, and the study is performed in conformance with the Declaration of Helsinki ethical guidelines (http://ohsr.od.nih.gov/helsinki.php3).

The computer-controlled freezer NiCool Plus PC (Air Liquide–DMC, Bussy Saint-Georges, France) is used for cryopreservation. In order to ensure rate-controlled freezing with a temperature decrease of 2°C/min (between 6°C and -40°C) and 5°C (between -40°C and -150°C), an optimized program is developed and adjusted accordingly.

As a cryoprotectant, a solution containing 50% DMSO (Cryo-Sure, WAK-Chemie, Dessau-Tornau, Germany) in HAES-steril 10% (Fresenius-Kabi AG, Bad Homburg, Germany) is used. Prior to freezing, a part of pre-cooled cryosolution (with 50% DMSO) is mixed with 3 parts of the buffy coat (pre-cooled) to achieve a DMSO concentration of 10% in the final solution. Cryopreservation is then carried out in aliquots of 3 mL in 3-mL cryogenic vials (Brand, Wertheim/Main, Germany). After freezing, the tubes are stored permanently in the liquid phase nitrogen for at least 2 weeks before thawing and analysis. This is to ensure the cells are fully settled to this temperature.

Following cryotube thawing, which is carried out in a water bath at 37°C, the sample is washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS, Invitrogen, Paisley, UK). Subsequently, the cell pellet is resuspended in 1 mL DPBS at room temperature.

Trypan Blue Exclusion Test

Prior to staining, 10 µL of the cell suspension is transferred to a sterile 1.5 mL tube. An equal volume of 0.4% Trypan blue in 0.85% saline solution (ie, 10 µL) is added to the cell suspension, and the mixture is incubated at room temperature for 1-2 min. A glass coverslip is placed on the counting chambers of an improved Neubauer hemocytometer, and the mixture is loaded onto 1 chamber so that the mixture exactly fills the chamber. The 4 large corner squares of the chamber are observed under a light microscope, and the viable/live (clear) or non-viable/dead (blue) cells are recorded for each square.[14]

The cell count is derived from the formula (# of live cells in 4 corner squares/4) × dilution factor (ie, 2) × 10^4 cells/mL. The viability is recorded for each corner square using the formula (# of live cells/total # of cells counted) × 100, and the overall viability is the mean of the 4 viabilities derived from the 4 corner square counts. These formulae are derived from the dimensions of each chamber of the hemocytometer, which are 1 mm × 1 mm × 0.1 mm. All reagents should be filtered through a 0.2 µm syringe filter and warmed to 37°C prior to usage.

Glo-Max CytoTox-Glo Procedure

All CytoTox-Glo Cytotoxicity assay components are thawed in a 37°C water bath and mixed to ensure homogeneity. The CytoTox-Glo Cytotoxicity assay reagent is prepared by transferring the contents of 1 bottle of assay buffer to the AAF-Glo substrate bottle. The lysis reagent is prepared by transferring digitonin to the assay buffer. Both solutions should be mixed well to ensure homogeneity.

The CytoTox-Glo Cytotoxicity assay reagent and the lysis reagent can be stored at 4°C for up to 7 days with no appreciable loss of performance.

Prior to the evaluation of the viability of thawed hematopoietic cells, a primer experiment is carried out to evaluate assay linearity. We used a hemocytometer to obtain 100,000 viable cells/mL in at least 3.0 mL of fresh medium. We added 100 µL of the 100,000 cell/mL suspension (10,000 cells/well) to all wells of rows A and B in a 96-well plate. One hundred microliters of fresh medium was added to all wells in rows B-H. Fifty microliters of lysis reagent was added to all wells of columns 7-12 to lyse cells; these were the treated samples. Fifty microliters of the remaining assay buffer was added to all wells in columns 1-6 so that all wells had equal volumes; these were the untreated samples. Fifty microliters of the CytoTox-Glo Cytotoxicity assay reagent was added to each well. The plates were incubated at room temperature for 15 min.

To evaluate the viability of thawed hematopoietic cells, 5 µL of the cell suspension is transferred to 995 µL DPBS and mixed well. One hundred microliters of this new suspension is then transferred to a white 96-well plate and 50 µL AAF-Glo Substrate is added, mixed well, and incubated in a dark chamber for 15 min. After this period, the Relative Luminescence Units (RLU) are measured, and the readings are displayed in a Microsoft Excel spreadsheet. Fifty microliters of the lysis reagent is added to the well and incubated in a dark chamber for 15 min,

after which period the final RLU (of all the lysed cells) is measured, and the reading is displayed in the same MS Excel spreadsheet. The viability percentage is calculated by subtracting the initial RLU from the final RLU and divided by the final RLU.[9]

Results

The linearity of the CytoTox-Glo is shown in **Figure 1**. The RLUs of both treated and untreated cells are directly proportional ($R^2 = 0.9979$ for the treated cells and $R^2 = 0.9987$ for the untreated cells) to the cell number. Therefore, we can evaluate the viability percentages using the RLU counts.

Calculated viability percentages for both CytoTox-Glo and Trypan blue assays are shown in **Table 1**, as well as the differences between the 2 assays.

Discussion

We propose an alternate, time-saving, and accurate assay for viability percentage evaluation using the CytoTox-Glo kit and the Promega GloMax luminometer. We evaluated the viability percentage of 100 thawed samples of hematopoietic progenitor cells using both the trypan blue exclusion test and the CytoTox-Glo Assay. Method comparison demonstrated that 85% of the total numbers of the differences were between -3% and 3%. With a total incubation time of 30 min and the viability evaluation of up to 96 samples in a 96-well plate at the same time, the total time for an accurate viability evaluation is greatly minimized.

A major advantage of UCB is that, unlike embryonic stem cells, it is not a subject of social and political controversy, and it is a non-controversial source of post-natal stem cells.[15,16]

The UCB progenitor cells have high plasticity and proliferation capacity.[17] The more we learn about the biology of these cells and how they can be manipulated for self-renewal, survival, proliferation, differentiation, and migration/homing/mobilization, the more likely we are to use this information for clinical efficacy and benefit.[18]

Hematopoietic progenitor cells are routinely cryopreserved in liquid nitrogen at temperatures of -196°C; at these low temperatures almost all biological functions are shut down. The blood is stored at this temperature until it is ready to be used; this process involves rapid blood thawing in a water bath at 37°C before being administered to the patient. The processes that cause most cell damage are freezing and thawing. In order to reduce the damaging effect of cryopreservation, DMSO is used. Dimethyl-sulphoxide is an intracellular cryoprotectant displacing water from within the cell and thus reducing the formation of ice crystals that would otherwise damage it. Dimethyl-sulphoxide, however, is toxic to cells when exposed to temperatures above freezing. Dimethyl-sulphoxide has also been associated with adverse effects in transplant recipients.[19]

Cell viability is an important issue when assessing cell doses in cord blood units for transplantation. Generally, trypan blue staining is used in most centers.[4] However, the trypan blue exclusion test has several major disadvantages. Hemocytometer readings are subjective and therefore possess significant accuracy errors. Since it is a highly subjective assay, there is a significant variation of hemocytometer viability results among different laboratory personnel when analyzing the same cell sample. [9]

Another automated system is available for the viability evaluation using trypan blue. The Vi-Cell (Beckman Coulter) removes the subjectivity inherent in the method by providing an objective analysis of cellular viability. The Vi-Cell provides data and cleans the flow cell in less than 3 mins. Additionally, the Vi-Cell tests 15 to 30 times the sample volume of a hemocytometer. The larger

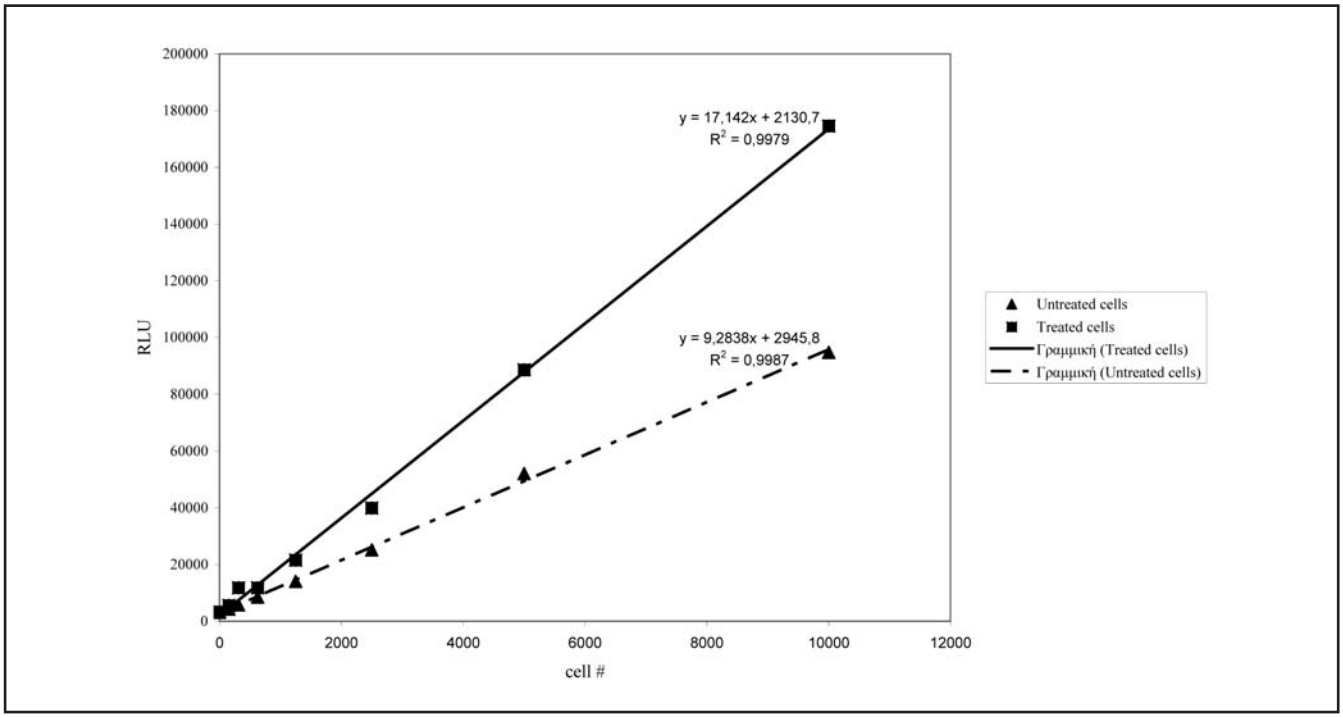

Figure 1_Linearity of CytoTox-Glo Assay of Treated Cells (using Lysis Reagent) and Untreated Cells (Without Using Lysis Reagent).

Table 1_Viability Percentage Using the Two Assays Performed

	CytoTox-Glo	Trypan Blue	Viability Differences (%) (CytoTox-Glo Minus Trypan blue)
N	100	100	100
Mean viability (%)	91.91	92.21	0.29
S.E.	0.403	0.388	0.282
S.D.	4.033	3.875	2.815
Minimum	77	75	-9
Maximum	98	98	6
Variance	16.265	15.016	7.925

number of cells included in the analysis provides much greater statistical confidence than results from the manual method. The instrument uses as little as 0.5 mL of sample volume.[9] This system facilitates the procedure, but it measures a single sample at a time. Furthermore, it needs larger amounts of cell suspension aliquots (0.5 mL) and a CCD camera. CytoTox-Glo Assay has the ability to evaluate 96 samples in a single reading session using a luminometer and a PC.

Keywords: Cell viability, CytoTox-Glo, trypan blue exclusion test, umbilical cord blood

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