The prats and pitfalls of assessing cell viability after cryopreservation

Brian J. Hawkins[1,3], Alireza Abazari[1], Faith Kalucki[2], Benjamin Fryer[2], Aby J. Mathew[1]

[1]BioLife Solutions, Bothell, WA, [2]Heart Regeneration Program, University of Washington, Seattle, WA, [3]Department of Anesthesiology and Pain Medicine, University of Washington, Seattle, WA

BioLife Solutions®
BIOPRESERVATION TOOLS FOR CELLS, TISSUES, AND ORGANS

Heart Regeneration Program
UW Medicine

ISCRM Institute for Stem Cell & Regenerative Medicine at the University of Washington

ABSTRACT

Technological advances have transformed cells from mere drug targets into potent 'living drugs' with the potential to cure formerly incurable diseases such as cancer. However, 'living drugs' require more complex logistical support to successfully maintain their efficacy during storage and transportation, and are commonly frozen in a process known as cryopreservation. Successful cryopreservation ensures optimal recovery, viability, and return to function post-preservation. In contrast, non-optimized cryopreservation exerts undue stresses during the freezing and thawing process that can lead to excessive cellular dysfunction and death, possibly rendering a cell dose ineffective. To evaluate cryopreservation efficacy, viability is commonly determined using markers of structural integrity immediately post-thaw and those values used as a benchmark for dosing. However, structural integrity markers in cryopreservation-stressed cells may not accurately reflect long-term viability and functionality in patients. It is therefore absolutely essential that rapid, accurate, and consistent methods of evaluating cell health be incorporated into the product lifecycle prior to patient delivery to ensure effective dosing and clinical effectiveness. In this study, our group demonstrates the inaccuracy membrane integrity-based assays, and propose alternative means of evaluating post-cryopreservation cell health that may be more representative of long-term viability and functionality.

METHODS

Cell Culture: The Jurkat (Clone E6-1) human acute T-cell leukemia (ATCC, Manassas, VA) was cultured in RPMI 1640 (Lonza, Walkersville, MD) supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO). The ELF1 human Embryonic Stem Cell line (University of Washington, Seattle, WA) was cultured in an albumin-free, chemically defined, E8™ medium (ThermoFisher Scientific, Waltham, MA).

Cryopreservation: Jurkat and ELF1 cells were cryopreserved in the indicated cryomedia, placed in 1.2 or 2.0 ml cryovials, and cooled at 2-8°C for 15 min. Cryovials were frozen using either an isopropanol freezing device or controlled rate freezer, and then transferred to LN_2 storage for a minimum of 24 hr. Samples were thawed in a 37°C water bath, immediately resuspended in culture media (1:10 dilution), and then either plated on laminin-coated culture vessels (hESCs) or cultured in suspension (Jurkat). The ELF1 hESC line was cryopreserved in CryoStor® cryomedia at increasing DMSO concentrations at a density of ~$1x10^6$ cells/m in the presence of the ROCK1 inhibitor Y-27632. Following liquid phase LN_2 storage, cells were thawed in a 37°C water bath and then resuspended at a ratio of 1:10 in Complete Growth Medium (CGM) without post-thaw wash to remove residual DMSO.

Mitochondrial Function: Mitochondrial membrane potential was evaluated immediately post-thaw and at indicated time points using the JC-1 ratiometric fluorophore and fluorescence recorded on the NC-3000 imaging cytometer (ChemoMetec).

Viability & Recovery: Structural cell viability was evaluated immediately post-thaw and at indicated time periods via membrane integrity using either the NucleoCounter NC-100, NC-200, or NC-3000 and a Via-1 cassette (ChemoMetec).

Apoptosis and DNA Fragmentation: Post-cryopreservation caspase 3/7 activity was determined in Jurkat T-cells at the indicated time point using the NucView488™ fluorescent substrate and the NC-3000 equipped with the Flexicyte package. Results were normalized to non-frozen controls. To determine DNA fragmentation, cells are incubated with a lysis buffer containing DAPI for 5 min at 37°C and DNA content measured using the NucleoCounter NC-3000.

Cellular Glutathione: Cellular thiol content (glutathione) was determined using the VitaBright-48 bimane reagent and the NucleoCounter NC-3000.

Statistical Analysis: For all measurements, data represent the mean of 3-14 independent experiments and are normalized to pre-freeze conditions and 37°C controls as indicated. Groups were analyzed for statistical significance via two-way ANOVA with Tukey post-hoc comparisons and $p<0.05$ set *a priori*. Error bars represent standard error of the mean (SEM).

Abbreviations: Ctrl – culture control, CGM – Complete Growth Medium, CSB – CryoStor® CSB, CS2 – CryoStor® CS2, CS5 – CryoStor® CS5, CS10 – CryoStor® CS10, DMSO – dimethylsulfoxide, $\Delta\psi_m$ – Mitochondrial Membrane Potential, GMP – Good Manufacturing Practices, GSH – glutathione, HBSS – Hank's Balanced Salt Solution, hESC – human Embryonic Stem Cell, HSA – human serum albumin, PLA – PlasmaLyte A

CONCLUSIONS

1. Measures of immediate post-thaw membrane integrity can vary over time and may not accurately reflect long-term cell viability following cryostorage. Additional measures of cellular function should be employed to more accurately assess cell health following cryopreservation.
2. Release criteria should include post-thaw measures of functional viability to accurate reflect the therapeutic effectiveness of cell therapies.
3. Cryopreservation stress negatively impacts mitochondrial function. Loss of mitochondrial membrane potential post-thaw accurately reflects long-term cell viability and may serve as a surrogate indicator of post-thaw cell health.
4. Altered cellular redox balance can be detected rapidly post-thaw and suggest oxidative stress following cryopreservation. Oxidative stress and decreased GSH levels may contribute to cryopreservation-induced cell death/damage.
5. The onset of cryopreservation-induced apoptosis can be accurately detected within hours post-thaw by the measurement of caspase 3/7 activity and DNA fragmentation. The presence of apoptotic indicators corresponds to the observed decline in long-term cell viability as determined by membrane integrity.
6. Measures of post-thaw cell viability and functionality are highly dependent upon the cryomedia employed. Optimized cryomedia improves viable recovery versus traditional 'home-brew' cryomedia, reduces cryopreservation-induced apoptosis, and conveys more accuracy to membrane integrity as an early indicator of cell viability.
7. These data stress the need for multiple measures of cell viability post-thaw, and suggest that cryomedia optimization is a vital component of cell therapy manufacturing. Cryopreservation optimization facilitates the accurate evaluation of post-thaw viability from master and working cell banks across multiple post-thaw viability assessment protocols.

RESULTS

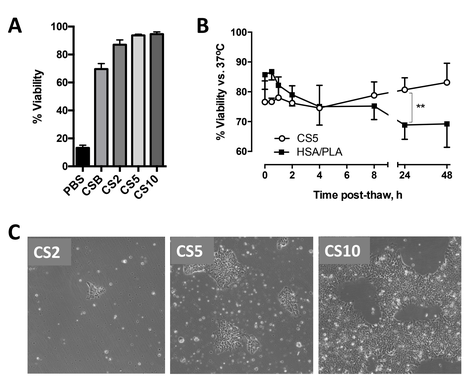

Figure 1. Cell membrane integrity immediately post-thaw is not representative of long-term cell health. (**A**) ELF1 hESCs exhibited elevated post-thaw cell viability in CryoStor® media with increasing DMSO content as assessed by the Chemometec NucleoCounter NC-100 versus PBS control. (**B**) Microscopic evaluation of hESC morphology 72 hr post thaw reveals disparate cluster formation that diverges from immediate post-thaw viability measurements. (**C**) Jurkat T cells cryopreserved in either a traditional T-cell cryomedia composed of PlasmaLyte A supplemented with 10% human serum albumin and 5% DMSO (HSA/PLA) or CryoStor® media (CS5) were thawed and cell viability evaluated immediately post-thaw and at increasing intervals up to 48 hr using the Via-1 cassette and visualized by the Chemometec NucleoCounter NC-3000 imaging cytometer. Jurkat cells cryopreserved in traditional HSA/PLA cryomedia experienced a significant loss in viability (**p<0.001) at 24 hr versus cells cryopreserved in CS5 cryomedia.

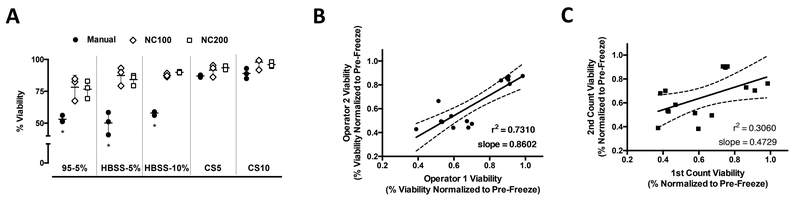

Figure 2. Membrane integrity-based post-thaw viability exhibits process-dependent variability. Jurkat cells cryopreserved at a density of $5x10^6$/ml in the indicated cryomedia were thawed and viability assessed using both automated (NC-100 and NC-200) and manual (Trypan Blue) methodologies. Manual counting was performed independently by two blinded and trained technicians. (**A**) Trypan Blue manual counting exhibited a significant and cryomedia-based deviation from automated methods (*p<0.0001 Manual vs. NC-100 & NC-200). Post-hoc correlational analysis of Trypan Blue viability measurements reveal the influence of both (**B**) technician identity and (**C**) count order on a manual counting protocol. These data suggest indicate the need for multiple measures of cell viability post-thaw, and suggest that cryomedia selection has an influence on post-thaw viability.

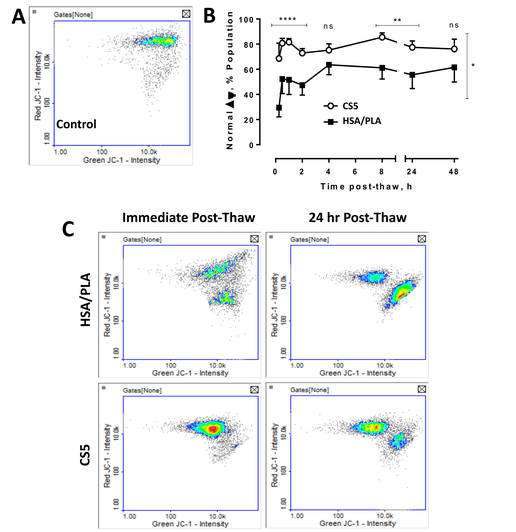

Figure 3. Cryopreservation induces persistent alterations in mitochondrial membrane potential ($\Delta\psi_m$). $\Delta\psi_m$ recovery is crucial for ATP production and a return to function of cells post-thaw. Delayed or absent $\Delta\psi_m$ recovery indicates dysfunctional mitochondria which may lead to diminished cell functionality or programmed cell death through mitochondrial-directed apoptosis. Cryopreserved Jurkat cells were thawed and stained for $\Delta\psi_m$ using the ratiometric fluorophore JC-1. Aggregation of green fluorescent JC-1 within a polarized mitochondrial membrane causes a red fluorescence shift. (**A**) JC-1 aggregate formation in functional mitochondria exhibit elevated red fluorescence. **(B)** Time course of post-thaw recovery of $\Delta\psi_m$ for Jurkat cells frozen in the indicated cryomedia reveal a rapid increase in $\Delta\psi_m$ post-thaw that persists for 24 hr post-thaw (* p<0.0005, ** p<0.005, *** p<0.05). **(C)** Representative image-cytometry graphs of mitochondrial membrane potential distribution in Jurkat T cells as assessed by JC-1 staining.

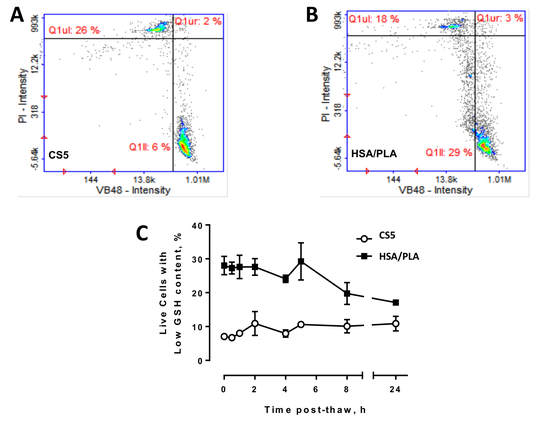

Figure 4. Cryopreserved cells exhibit altered cellular redox balance. Glutathione (GSH) is the largest contributor to the cellular thiol pool and is essential for numerous oxidation/reduction reactions. Mitochondrial dysfunction can result in excess generation of reactive oxygen species that dimerize two GSH molecules and reduce free GSH levels and cellular antioxidant capacity. To determine whether cryopreservation-induced mitochondrial dysfunction associates with oxidative stress, Jurkat T cells were evaluated immediately post-thaw and at increasing time intervals with the thiol-reactive fluorophore VB-48. Fluorescence dot-plots of VB-48 (free cellular GSH) versus Propidium Iodide (PI) (plasma membrane permeability) in Jurkat cells cryopreserved in (**A**) CS5 and **(B)** HSA/PLA cryomedia. Fluorescence intensity was normalized to non-frozen controls as indicated. **(C)** Cryopreservation resulted in an increased cell population with low thiol (GSH) content versus non-frozen controls that was dependent upon the cryomedia employed.

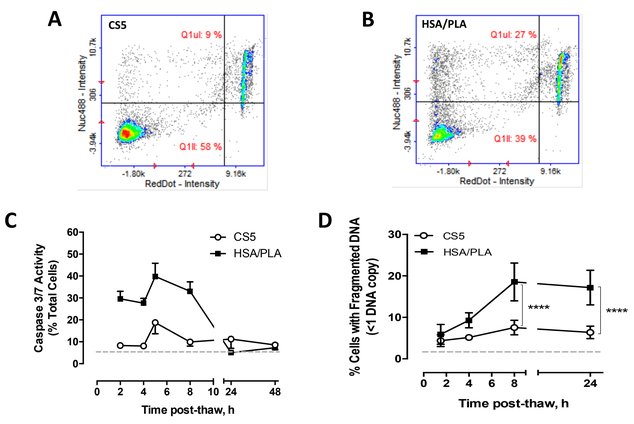

Figure 5. Cryopreservation-induced apoptosis contributes to the post-thaw decline in cell viability. To determine the mechanism by which cryopreservation associates with a decline in cell viability at 24 hr, Jurkat cells cryopreserved in the indicated cryomedia were evaluated for caspase 3/7 activity at incremental time points. (**A**) Caspase 3/7 activity peaked 2 hr post-thaw in Jurkat cells that normalized following 24 hr of post-thaw culture. Representative image cytometry dot-plot of fluorescence intensity of the caspase 3/7 fluorescence substrate (Nuc488™) and the necrotic indicator RedDot in Jurkat cells cryopreserved in (**B**) CS5 and (**C**) HSA/PLA. Fluorescence intensity was normalized to non-frozen controls as indicated. **(D)** Cell cycle analysis using DAPI revealed a significant increase in Jurkat T cells exhibiting fragmented DNA that corresponded with the downstream effects of caspase 3/7 activity. Dashed lines in **(C)** and **(D)** represents caspase 3/7 activity and % cells with fragmented DNA in non-frozen controls, respectively. (****p<0.0001)