The Effect of Cryomedia Selection and Transient Warming Events on Post-Cryopreservation Human MSC Function

Brian J. Hawkins[1,2], Alireza Abazari[1], Lye Theng Lock[3], Taby Ahsan[3], Jon A. Rowley[3], John Fink[4], Aby J. Mathew[1]

[1]BioLife Solutions Inc., Bothell, WA, [2]University of Washington, Seattle, WA, [3]RoosterBio Inc., Frederick, MD, [4]Brooks Life Sciences, Chelmsford, MA

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

RoosterBio

Brooks LIFE SCIENCE SYSTEMS

ABSTRACT

Cellular therapies have proven clinically effective and have the potential to revolutionize medicine. Cryopreservation in GMP-grade cryomedia and storage and transportation in vapor phase liquid nitrogen (LN2) are the preferred commercialization methods for cellular therapies. Routine sample access of cell banks stored in LN2 may expose adjacent doses to temperature excursions that could negatively impact the stability of frozen cells. The purpose of this study was to assess the effect of two commonly employed cryopreservation medias and repetitive TWE on clinically-relevant Mesenchymal Stem/Stromal Cells (MSCs), and determine how the incorporation of evidence-based Best Biopreservation Practices can improve cellular outcome.

Xeno-free human MSCs (RoosterBio, Frederick, MD) were cryopreserved either in a traditional home-brew MSC cryomedia (HB; 5% Human Serum Albumin/10% DMSO/Plasma-Lyte A) or in GMP-manufactured CryoStor CS5® cryomedia (CS5; BioLife Solutions, Bothell, WA). To mimic clinical conditions, cryopreserved samples were shipped in an evo® DV-4 LN2 smart shipper (Savsu Technologies, Albuquerque, NM) to Brooks Life Sciences (Chelmsford, MA). Upon receipt, samples were divided into separate cryoboxes for each experimental condition and stored in a LN2 vapor freezer (BioStore III Cryo automated freezer). Over two weeks, the cryoboxes were removed from the freezer to warm the samples to approximately -110°C for 0, 5, 10, 15, and 20 TWEs. Samples were then sent to RoosterBio and BioLife for determination of viability, expansion, IDO secretion, and metabolic activity both immediately post-thaw and following post-thaw culture.

Immediate post-thaw viability was similar between samples frozen in HB and CS5 with no overall trend observed between viability and TWE number. All cells experienced rapid post-thaw cell expansion in culture. Despite these apparent similarities, cellular metabolic activity (alamarBlue) was elevated in cells frozen in CS5 versus HB both immediately post-thaw and after 24 hr of culture, and a decline in MSC immunomodulatory activity was apparent at 20 TWE. These results suggest that both the cryomedia employed and the number of temperature excursions during storage affect functionality and potency of cryopreserved cellular therapies. As such, our findings emphasize the importance of optimized cryopreservation protocols and storage conditions to maximize the effectiveness of cellular therapies in patients.

METHODS

Cell Culture: Xeno-free human MSCs (hMSCs) (RoosterVial-hBM-20M-XF) were cultured in RoosterBasal® -MSC media supplemented with RoosterBooster® -MSC-XF in Corning® CellBIND® Surface CellSTACK® culture chambers.

Cryopreservation: hMSCs (1x10^6 cells/ml) were cryopreserved in either xeno-free CryoStor CS5® cryomedia (CS) or a traditional 'home-brew' cryomedia composed of PlasmaLyte A supplemented with 5% human serum albumin and 10% DMSO (HB). hMSCs suspended in cryomedia were placed in 1.2 mL FluidX® cryovials, and cooled at 2-8°C for 10 min. Cryovials were cooled at -1°C/min using either an isopropanol freezing device or Asymptote controlled rate freezer to -10°C, manually nucleated, and then frozen to -80°C. Cryovials were then transferred to LN_2 storage for a minimum of 24 hr.

Shipping & Storage: Cryovials were transported between facilities using a LN_2 dry shipper and temperature monitored using a data logger. Long-term LN_2 storage was conducted in a BioStore® III Cryo -190°C Storage System (Brooks Life Sciences, MA). Sample temperature was maintained during all handling periods using a CryoPod® carrier (Brooks Life Sciences, MA).

Transient Warming Events: The hMSC control group remained inside the LN_2 freezer, whereas the TWEs were initiated by temporarily exposing cryovials to the ambient environment 0, 5, 10, 15, and 20 times. In each TWE cycle, cryovials were exposed to the ambient environment reaching a temperature of approximately -110°C, and then returned to the LN_2 freezer. The TWE time to warm the samples to -110°C was approximately 10 min. Cryovials were allowed to return to below -180°C before the initiation of a subsequent TWE.

Viability & Recovery: Structural cell viability was evaluated immediately post-thaw and at indicated time periods via membrane integrity using the NucleoCounter® NC-3000™ imaging cytometer (ChemoMetec) and a Via-1 cassette.

Cell Functional Assessment: hMSC metabolism was measured at the indicated time periods using alamarBlue reagent and fluorescence measured on an Infinite® F200 (Tecan, Gmbh). Cells were imaged using a Zeiss Axiovert® inverted microscope. Mitochondria were visualized using MitoTracker Red and Mitochondrial Network Analysis (MiNA) determined via ImageJ.

Statistical Analysis: Unless indicated, data represent the mean of 6 independent experiments. Groups were analyzed for statistical significance via two-way repeated measures ANOVA with Tukey post-hoc comparisons and $p<0.05$ set *a priori*. Error bars represent standard error of the mean (.S.EM).

Abbreviations: CS – CryoStor® CS5, DMSO – dimethylsulfoxide, GMP – Good Manufacturing Practices,HB – 'home-brew', hMSC – human Mesenchymal Stem/Stromal Cells, IFN-γ – Interferon Gamma, TWE – Transient Warming Event

CONCLUSIONS

1. RoosterBio® BM-hMSCs can maintain robust cell expansion and immunomodulatory functionality following cryopreservation up to P5.
2. RoosterBio® BM-hMSCs are resistant to mild and slow storage temperature excursions (<20 TWE) up to -110°C when cryopreserved in either GMP-manufactured CryoStor® media or traditional 'home-brew' media containing an increased DMSO concentration.
3. Optimized intracellular-like CryoStor® CS5 provides comparable post-thaw viability to 'home-brew' cryomedia at a lowered cryoprotectant concentration (5% DMSO in CryoStor CS5 vs. 10% DMSO in 'home-brew').
4. Optimized CryoStor® media speeds the restoration of cell function following cryopreservation as evidenced by both cell adhesion/spreading during post-thaw culture and the significant improvement in cell metabolism at 48 hr post-thaw.
5. CryoStor® media facilitates the rapid restoration of hMSC mitochondrial structure post-thaw.
6. Membrane integrity-based measurements of post-thaw cell viability may not be indicative of hMSC health, and should be complemented with additional measures of cellular health immediately post-thaw.
7. These data suggest that optimized cryopreservation can improve post-thaw hMSC function and reduce the need for extended post-thaw 'rescue' culture, and may therefore improve the clinical efficacy of hMSC-based therapies.

RESULTS

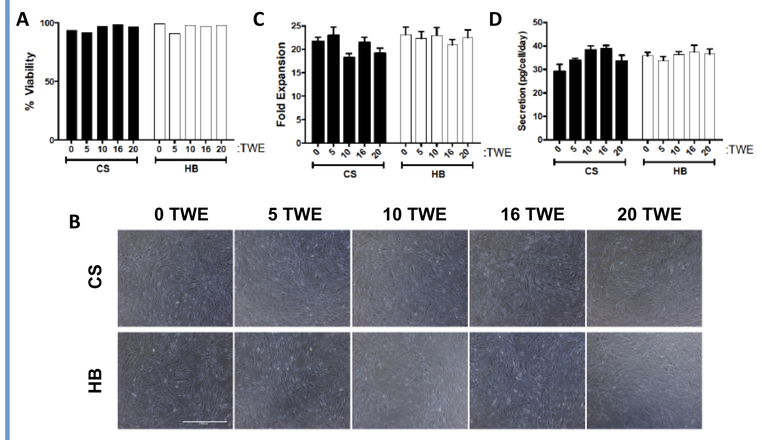

Figure 1. Transient warming events during storage do not impair long-term hMSC function. hMSCs cryopreserved at a density of 1x10^6/ml in either a traditional 'home-brew' cryomedia composed of PlasmaLyte A supplemented with 5% human serum albumin and 10% DMSO (HB) or GMP-manufactured CryoStor® media preformulated with 5% DMSO (CS) exhibit similar (**A**) viability post-thaw that is not influenced by repetitive transient warming events (TWE). After thaw, hMSCs (P3) were washed and plated at 3,000 cells/cm^2 in RoosterBio basal medium supplemented with Booster and cultured to P4 for 5 days and evaluated for functionality. hMSCs in extended culture exhibit typical **(B)** morphology and **(C)** expansion potential regardless of the cryomedia employed or TWE number. **(D)** hMSCs (P4) display immunomodulatory potential via the secretion of angiogenic cytokines in response to IFN-γ challenge. Figures represent a single data point for each condition.

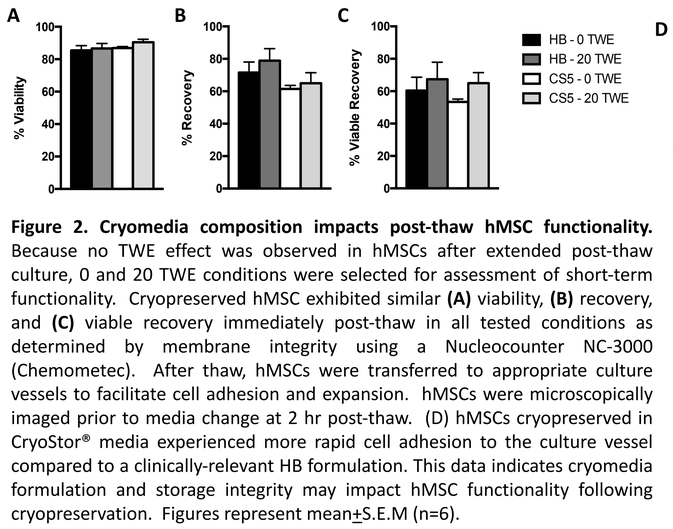

Figure 2. Cryomedia composition impacts post-thaw hMSC functionality. Because no TWE effect was observed in hMSCs after extended post-thaw culture, 0 and 20 TWE conditions were selected for assessment of short-term functionality. Cryopreserved hMSC exhibited similar **(A)** viability, **(B)** recovery, and **(C)** viable recovery immediately post-thaw in all tested conditions as determined by membrane integrity using a Nucleocounter NC-3000 (Chemometec). After thaw, hMSCs were transferred to appropriate culture vessels to facilitate cell adhesion and expansion. hMSCs were microscopically imaged prior to media change at 2 hr post-thaw. (D) hMSCs cryopreserved in CryoStor® media experienced more rapid cell adhesion to the culture vessel compared to a clinically-relevant HB formulation. This data indicates cryomedia formulation and storage integrity may impact hMSC functionality following cryopreservation. Figures represent mean±S.E.M (n=6).

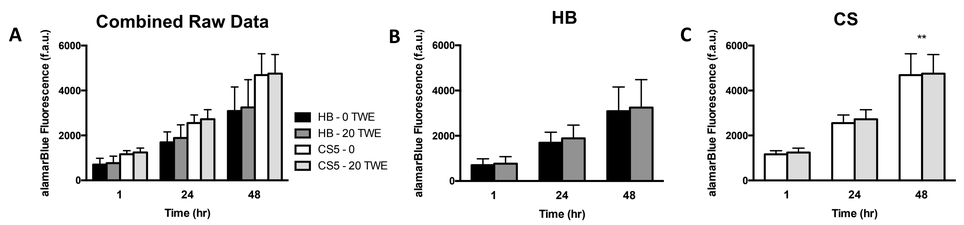

Figure 3. CryoStor® improves post-thaw cell metabolism. Post-thaw hMSC metabolic activity was assessed at 1 hr, 24 hr, and 48 hr post-thaw using alamarBlue® reagent, which is converted from nonfluorescent resazurin to fluorescent resorufin in metabolically active cells. (A) alamarBlue® fluorescence increased in all samples over 48 hr of post-thaw culture but was consistently higher in hMSCs cryopreserved in CryoStor® media. To reveal the influence of TWE number on hMSC metabolism over 48 hr, each cryomedia group was analyzed separately using 2-way repeated measures ANOVA (TWE x time). (B) hMSCs frozen in HB cryomedia exhibited high sample variability and no significant increase in cell metabolism over 48 hr in culture. (C) In contrast, hMSCs frozen in CryoStor® media demonstrated a highly significant (**$p<0.001$) increase in cell metabolism at 48 hr post-thaw. This data indicates that post-thaw hMSC metabolism is highly dependent upon the cryomedia employed during freezing. Figures represent mean±S.E.M (n=6).

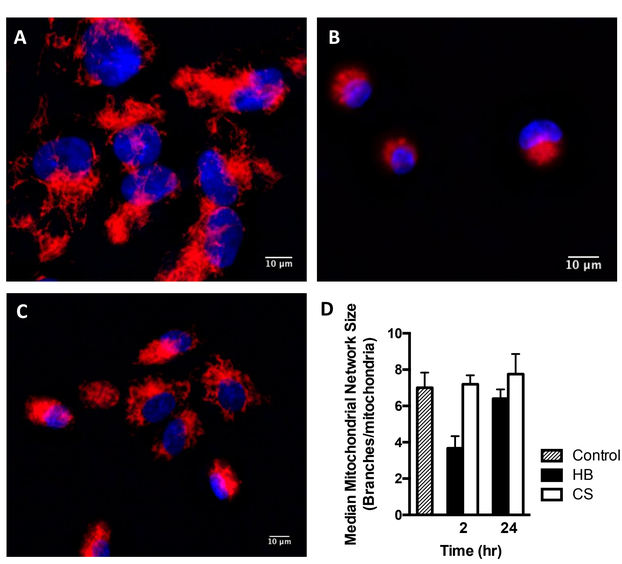

Figure 4. CryoStor® speeds the post-thaw restoration of mitochondrial morphology. Mitochondria are the primary site for intracellular energy production and metabolism. Mitochondrial structure ranges from a branched, reticulated network to punctate globules that differ between tissues and individual cell types. Fragmented, globular mitochondria are indicative of mitochondrial stress or dysfunction whereas a highly branched network is associated with more normalized mitochondrial metabolism and energy production. (**A**) Under normal culture conditions, undifferentiated hMSCs exhibit a predominantly reticulated and highly branched mitochondrial network (MitoTracker Red fluorescence; cell nuclei visualized with Hoechst 33342). (**B**) hMSCs cryopreserved in HB cryomedia exhibit a fragmented mitochondrial network indicative of cellular stress and altered metabolism 2 hr post-thaw. (**C**) In contrast, hMSCs frozen in CryoStor® CS5 display enhanced mitochondrial branching and a more normalized network 2 hr post-thaw. (**D**) Unbiased, automated image analysis of the mitochondrial network revealed that HB cryomedia requires extended post-thaw culture to normalize the mitochondrial network structure (represented as median branches/mitochondria). Conversely, hMSCs frozen in CryoStor® CS5 exhibit a numerically similar degree of mitochondrial branching at both 2 hr post-thaw and after extended culture. Mitochondria network analysis conducted on all cells in 3-5 microscopic fields. Figures represent mean±S.E.M.