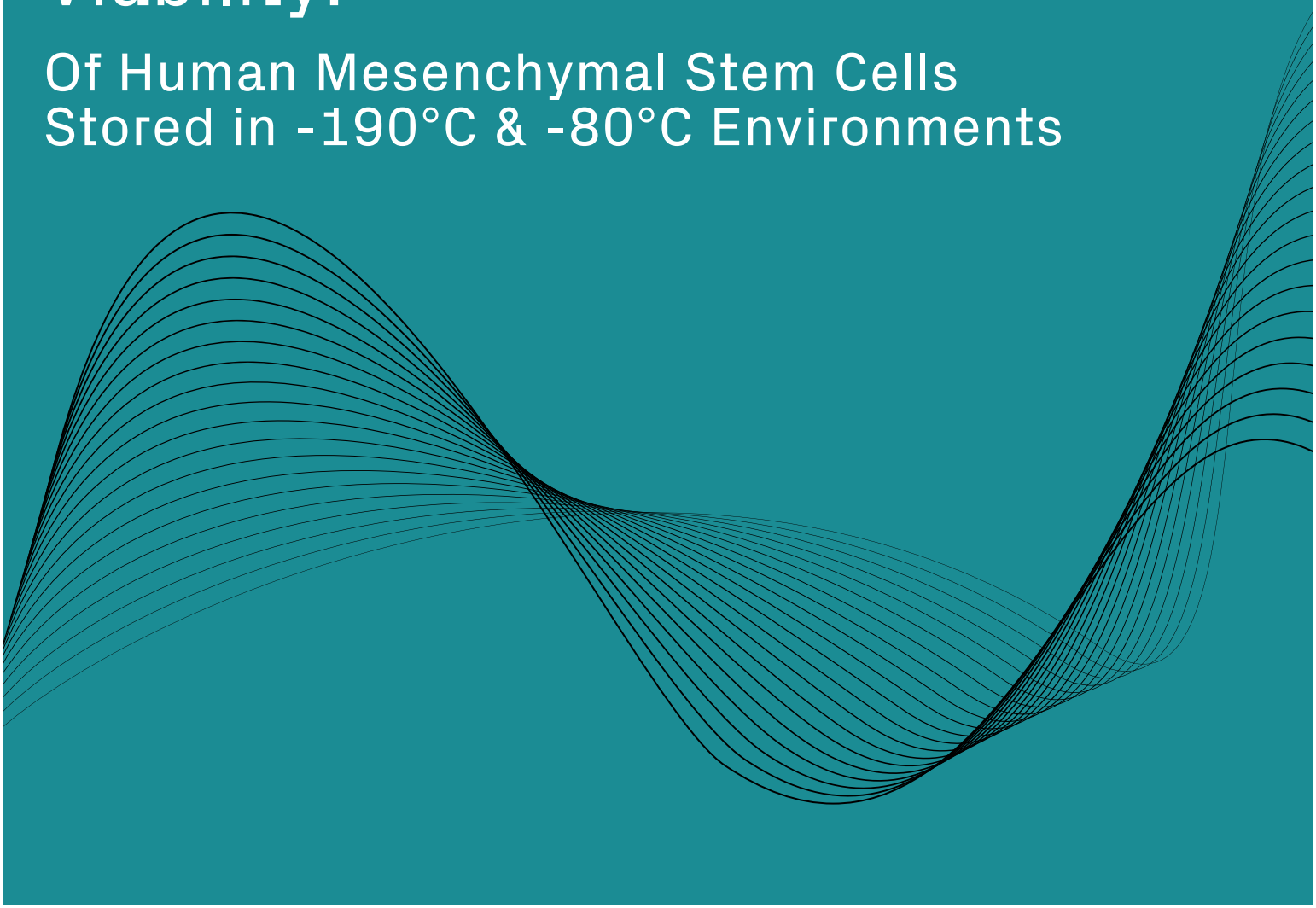
Storage, Automation & Logistics

WHITE PAPER

The Effects of Common Transient Warming Events on Post Thaw Recovery and Viability:

Of Human Mesenchymal Stem Cells Stored in -190°C & -80°C Environments



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Introduction

Cell Therapy products are normally transported and stored at or below -150°C in an LN2 vapor environment. Best practices typically recommend storing below the glass transition (Tg) of water, approximately -135°C. During routine sample access though, which is part of the normal life cycle of the product, thousands of adjacent (innocent) samples are exposed to ambient temperature for various, unmonitored durations. This exposure can represent a +200°C gradient between the freezer and the ambient environment. There is limited experimental research monitoring the effects these temperature excursions and the crossing of Tg have on the recovery and viability of therapeutic cells. Temperature cycling is believed to decrease cell viability as it induces thermal cycling stresses on the cells.

This paper's objective is to test and demonstrate the impact of both storage temperature and thermal cycling on the post thaw recovery and viability of human mesenchymal stem cells (hMSCs) over 15 months. In order to carry out these experiments a system was evaluated utilizing a closed-system cryogenic vial, -190°C cryogenic automated storage system, -80°C ULT freezer and a cryogenic carrier.

The results of this experiment after the first three months of storage were shown at ISCT 2016[a].

Materials

- Human Bone Marrow Derived MSC (hBM-MSC)
- 2mL CellSeal® Closed System Cryogenic Vial
- BioStore™ -190°C LN2-Based Automated Storage System (BioStore)
- CryoPod™ carrier
- TempAura™ remote temperature monitoring
- Biocision BioT™ ULT1.9 -80°C Freezer
- LN2 vapor shipper

Procedures

Cell Preparation and Shipping

hBM-MSC were purchased from RoosterBio Inc (USA). The cells were seeded at high density (1×105 cells/cm2) using High Performance Basal Media+XF Booster (RoosterBio Inc) to wake cells from cryopreservation induced stress. After two days in culture the cells were harvested using TrypLE™ Express (Invitrogen™, USA) cell dissociation reagent and pooled. The cell concentration and viability were assessed using Vi-Cell XR (Beckman Coulter, USA) and the cell number was adjusted to 2×106 cells/mL in complete cell culture media composed of DMEM-F12 with 10% Stemulate®.

The cryoprotectant medium (CPA) was prepared using 40% basal media (DMEM-F12) with 40% Stemulate® (Cook Regentec LLC, USA) and 20% DMSO (Origen biomedical, USA). The CPA was added gradually over a period of 5 mins to bring the final cell and CPA concentrations to 1×106 cells/mL and 10% DMSO with 25% Stemulate respectively. Next, the cells were aliquoted into CellSeal vials (Cook Regentec LLC, USA) at 1×106 Cells/vial and the tubing on the vials was sealed appropriately as per the manufacturer suggestions. The cells were frozen at a controlled rate (-1°C/min) using controlled rate freezing containers (Cook Regentec LLC, USA) overnight in a -80°C freezer. Next day, half of the vials were plunged into a liquid nitrogen vapor storage tank for storage at -190°C and the other half remained in the -80°C freezer. After one week the vials were shipped in two batches to Azenta Life Sciences (Chelmsford, MA). The -190°C samples in an LN2 dry shipper and the -80°C samples in an EPS shipper with 5kg of dry ice.

Storage and Controlled Warming Exposures

When received at Azenta Life Sciences, the two shipments of samples were divided into four batches (Fig 1). The two control batches at -190°C and -80°C were stored in, and never removed from, their respective storage freezers until the end of the experiments. The two variable batches were temperature cycled at random intervals over 13 months (never cycled more than once per 24hrs). The -190°C exposures were performed by lifting the rack part way out of the LN2 freezer for approximately 4 mins to only expose the cryobox with the variable set of CellSeal vials. The sample temperatures during these exposures was known from having a thermocouple in a separate CellSeal vial filled with water stored with the MSC samples. Monitoring this water filled vial during random exposures showed the samples warmed to between -100°C to -120°C during each exposure (Fig 2). Room air flow as well as frost accumulation both contributed to the rate of warming and thus, final temperature after a 4 min exposure. The samples from the -80°C freezer to LN2 freezer (and back) were always transported in the CryoPod carrier at -180°C or colder to eliminate any warming during transport between freezers. After 3 and 13.5 months of storage, 3 vials from each batch were picked at random and shipped back to Cook Regentec.

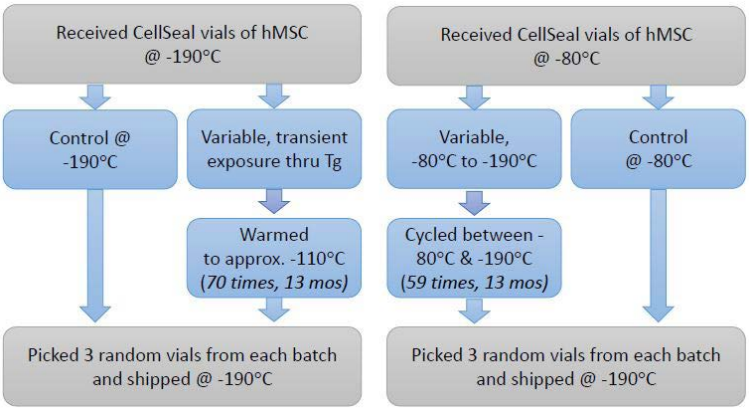


Figure 1: Sample exposure workflows for the four batches

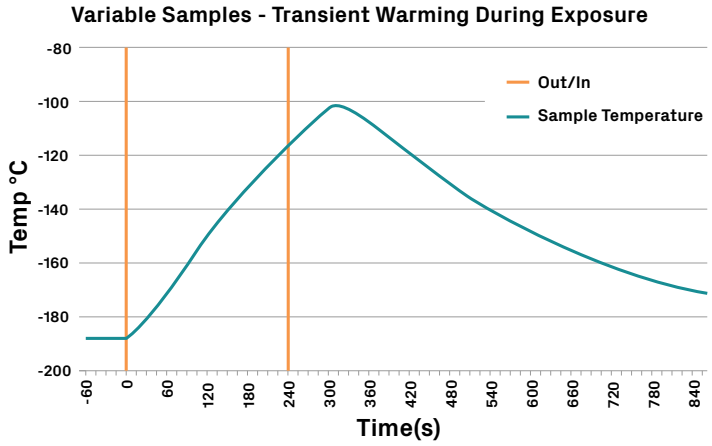


Figure 2: Example warming curve for -190°C exposed batch. Note, this warming rate is slower than typical, because only the top of the rack was exposed for these experiments. An innocent 2mL sample can cross Tg from -190°C in ~120 seconds when the entire rack is lifted from the LN2 freezer.

Thawing and Testing

Upon receipt at Cook Regentec, the vials were thawed in a water bath at 37°C until the last of the ice had just melted. To wash the cells and prepare them for viability testing, the suspension was initially diluted with complete cell culture medium (DMEM-F12 with 10% Stemulate) at a 1:1 v:v ratio over 10 min. Following this stepwise addition, the suspension was diluted with a second 1:1 v:v addition of complete culture medium (DMEM-F12 with 10% Stemulate). The suspension was centrifuged at 400g for 5 mins and then re-suspended in fresh complete medium to complete the washing process.

Immediate post-thaw viability and recovery were measured using a standard trypan blue dye exclusion assay. Briefly, 10µl of concentrated cell suspension from a representative vial from each batch was mixed with an equal volume of trypan blue (Sigma, USA) in an Eppendorf (Corning, USA) tube. Next, 10µl of dyed cell suspension was pipetted into the Hemocytometer counting chamber. Cells stained blue were considered non-viable, clear cells were considered viable. To measure functionality, proliferation rate was measured using an ATP luminescence assay. The ATP levels were measured on Day 0 through Day 4 using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA) following the manufacturer's protocol. Luminescence was read using the BioTek™ Synergy™ NEO2S Multi-Mode Microplate Reader using the default luminescence settings. Furthermore, the thawed cells were re-examined by flow cytometry to evaluate any change in cell-specific markers due to ambient exposure.

Results

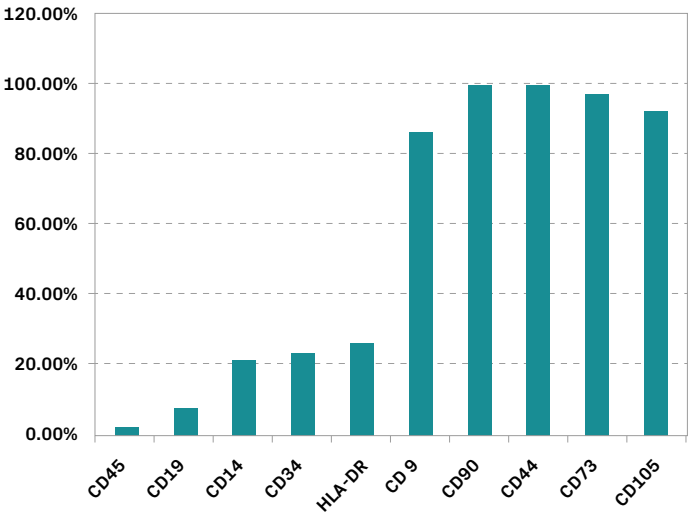
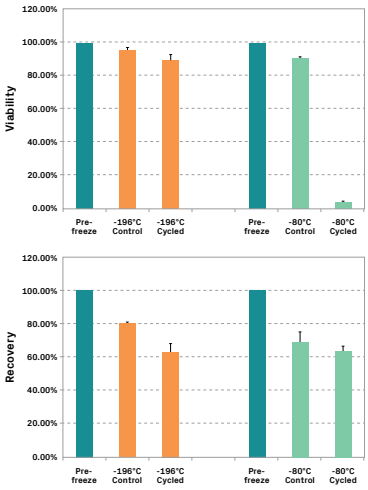


Figure 3: Pre-freeze fluorescence-activated cell sorting (FACS) analysis for surface antigen profiling of hBM-MSCs. Surface expressions were analyzed by using a BD FACS Caliber instrument with BD Cell Quest™ software.



% Viability	
Pre-freeze	99.4% (± 1.0)%
-196°C Control	94.6 (± 0.9)%
-196°C Cycled	86.5 (± 2.5)%
-80°C Control	90.3 (± 0.4)%
-80°C Cycled	3.8 (± 0.2)%

% Recovery	
Pre-freeze	100%
-196°C Control	80.5 (± 0.8)%
-196°C Cycled	63.9 (± 4.9)%
-80°C Control	69.9 (± 5.6)%
-80°C Cycled	64.7 (± 2.6)%

Figure 4: The Viability and Recovery of hBM-MSC pre-freeze and postthaw for control and variable samples. Lower viability and recovery were observed for the -190°C cells that were temperature cycled vs -190°C control.

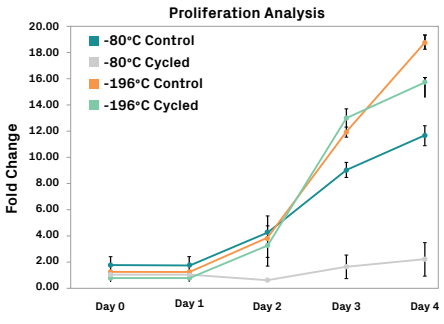


Figure 5: The proliferation of hBM-MSC stored at -190°C and -80°C for control and cycled samples. Data shows that the cells stored at -190°C proliferate more rapidly than the cells stored at -80°C.

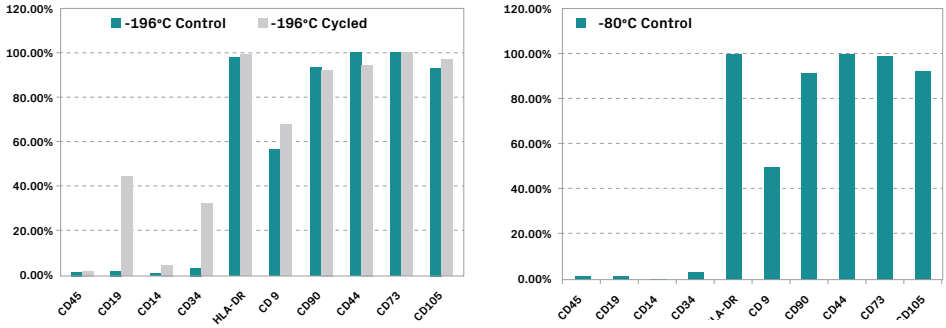


Figure 6: Post-thaw fluorescence-activated cell sorting (FACS) analysis for surface antigen profiling of hBM-MSCs. Surface expressions of CD45, CD19, CD14, CD34, HLA-DR, CD9, CD90, CD44, CD73 and CD105 were analyzed by using a BD FACS Caliber instrument with BD Cell Quest™ software. Prefreeze hBM-MSCs are shown in Fig 3.

Conclusions

- hBM-MSC stored at -190°C that repeatedly, though briefly, crossed the glass transition temperature (Tg -135°C H20) show reduced recovery, viability and proliferation than the control group that did not warm to Tg.
- hBM-MSC cells stored at -190°C have higher vitality, recovery and proliferation than the same cells stored at -80°C.
- These results are similar to the same experiments performed after the first three months of storage which was shown at ISCT 2016[a].

Original material presented at ISCT 2017 by:
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References:

1 Azenta Life Sciences
2 Cook Regentec

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