White Paper

THE EFFECT OF STORAGE TEMPERATURE AND REPETITIVE TEMPERATURE CYCLING:

ON THE POST THAW FUNCTIONALITY OF HUMAN MESENCHYMAL STEM CELLS

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INTRODUCTION

Stem cells are normally transported and stored at either -80°C or below -150°C in LN2 vapor. Best practices typically recommend storing below the glass transition (Tg) of water, approx -135°C. In industry however, there is discussion about, but limited experimental research surrounding cell recovery/viability of -80°C vs. -190°C. Additionally, at either storage temperature samples are typically repeatedly exposed to the ambient environment when adjacent samples are accessed. This temperature cycling is believed to decrease cell viability as it induces thermal cycling stresses on the cells. Due to many variables affecting post thaw functionality, standardization should be used where possible, such as cells should be processed and stored in a closed system and have their temperature, transient exposures and access controlled and monitored. This paper's objective is to demonstrate the impact of both storage temperature and thermal cycling on the post thaw viability and functionality of human mesenchymal stem cells (hMSCs).
In order to carry out these experiments, a system was evaluated utilizing a closed-system cryogenic vial (CellSeal), -190°C cryogenic automated storage system (BioStore III Cryo), -80°C ULT freezer and a cryogenic transport (CryoPod).

MATERIALS

- Human Bone Marrow Derived MSC (hBM-MSC)
- 2mL CellSeal® Closed System Cryogenic Vial
- BioStore™ III Cryo -190°C storage system (B3Cryo)
- CryoPod™ carrier
- TempAura™ remote temperature monitoring
- Biocision BioT™ ULT1.9 -80°C Freezer
- LN2 vapour shipper
- EPS shipper

PROCEDURES

Cell Preparation and Shipping

hBM-MSC were purchased from RoosterBio Inc (USA). The cells were seeded at high density (1x105 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 2x106 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 (approximately -1°C/min) using controlled rate freezing containers (COOK Regentec LLC) 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 Brooks Automation (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 Brooks Automation, 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 20 times over 67 days (2-3 times/week). The -190°C exposures were performed by lifting the rack part way out of the LN2 freezer for 4 mins each time, to expose the cryobox with CellSeal vials. The sample temperatures during these exposures was known from having a separate CellSeal vial with 2mL of water and a thermocouple installed in it. This vial was stored with the MSC samples. Monitoring this water filled vial during random exposures (n=5) showed the samples warmed to approximately -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 67 days of storage, 3 vials from each batch were picked at random and shipped back to Cook Regentec. The 6 from -190°C storage where shipped in an LN2 vapor shipper and the 6 from -80°C storage were shipped in an EPS with 5kg of dry ice.

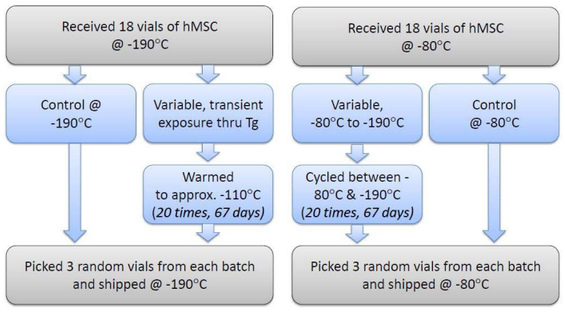


Figure 1.

The Effect of Storage Temperature and Repetitive Temperature Cycling on the Post Thaw Functionality of Human Mesenchymal Stem Cells

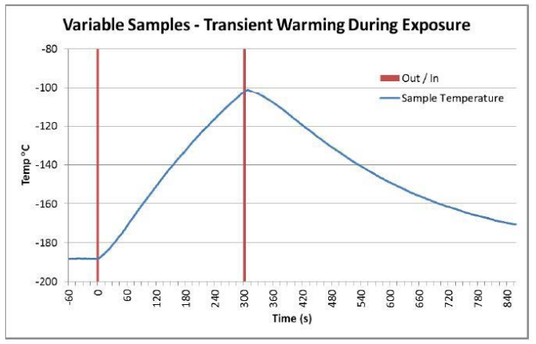


Figure 2.

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, Day 1, Day 2 and Day 3 using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA) following the manufacturer's protocol. Luminescence was read using the Biotek Synergy 2 plate 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

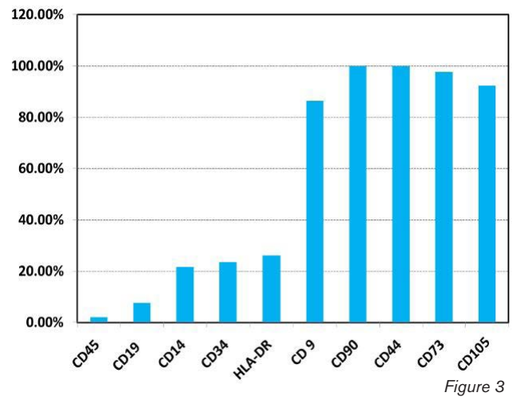
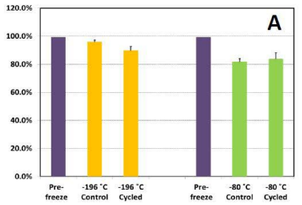
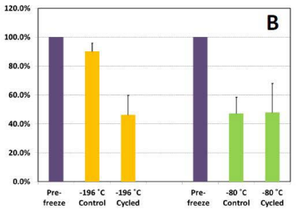


Figure 3

Figure 3. Pre-freeze 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. Pre-freeze hBM-MSCs strongly expressed the MSC markers CD105, CD73, CD9, CD90 and CD44 and were negative for the hematopoietic markers CD45, CD34, CD14, CD19 and HLA-DR.



% Viability	
Pre-freeze	99.4% (±1.0)%
-190˚C Control	96.0 (±1.4)%
-190˚C Variable	89.8 (±3.03)%
-80˚C Control	81.9 (±2.2)%
-80˚C Variable	83.9 (±4.2)%



% Recovery	
Pre-freeze	100.0%
-190˚C Control	90.2% (±5.8)%
-190˚C Variable	46.2 (±13.7)%
-80˚C Control	47.2 (±11.41)%
-80˚C Variable	48.1 (±20.0)%

Figure 4. The Viability (A) and Recovery (B) of hBM-MSC pre-freeze and post-thaw for control and variable samples. Lower viability and recovery were observed for the -190°C cells that were temperature cycled when compared to the -190°C control. For the -80°C samples, no significant difference in viability or recovery was noticed between both control and cycled samples.

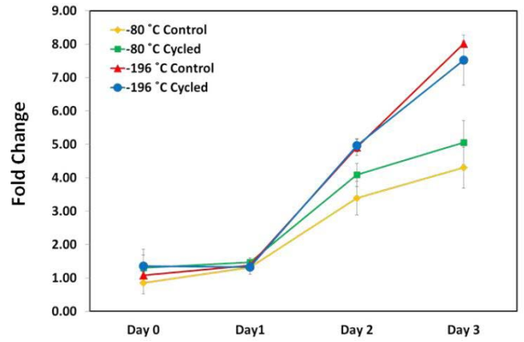


Figure 5. The proliferation of hBM-MSC stored at -190°C and -80°C for control and cycled samples. Data shows that the cells stored at -190°C proliferate more rapidly than the cells stored at -80°C.

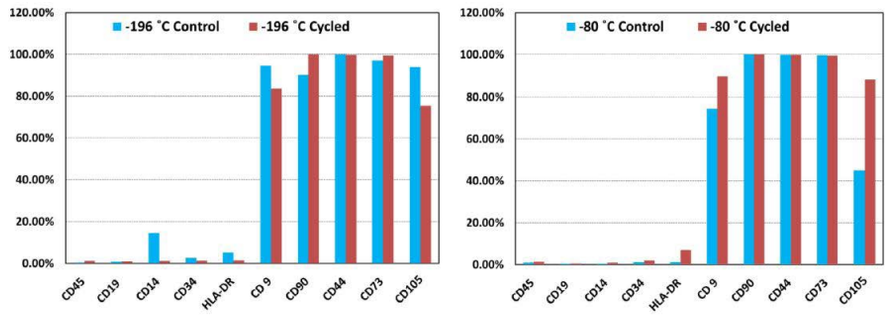


Figure 6. Post-thaw fluorescence-activated cell sorting (FACS) analysis for surface antigen profiling of hBM-MSCs. Surface expressions of CD45, CD19, CD14, CD34, HLA-DR, CD9, CD90, CD44, CD73 and CD105 were analyzed by using a BD FACS Caliber instrument with BD Cell Quest™ software. Pre-freeze hBM-MSCs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

CONCLUSIONS

- hBM-MSC cells stored at -190°C have higher vitality, recovery and proliferation than the same cells stored at -80°C.
- hBM-MSC stored at -190°C that repeatedly, though briefly, cross the glass transition temperature (Tg -135°C H20) show significantly reduced recovery than the same cells that did not warm past Tg.
- These experiments are ongoing with plans to test more cells after six and twelve months of storage and exposures.

Original material presented at ISCT 2016 by:
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