Delineating the Critical Process Parameters for Cell Therapy Cryopreservation

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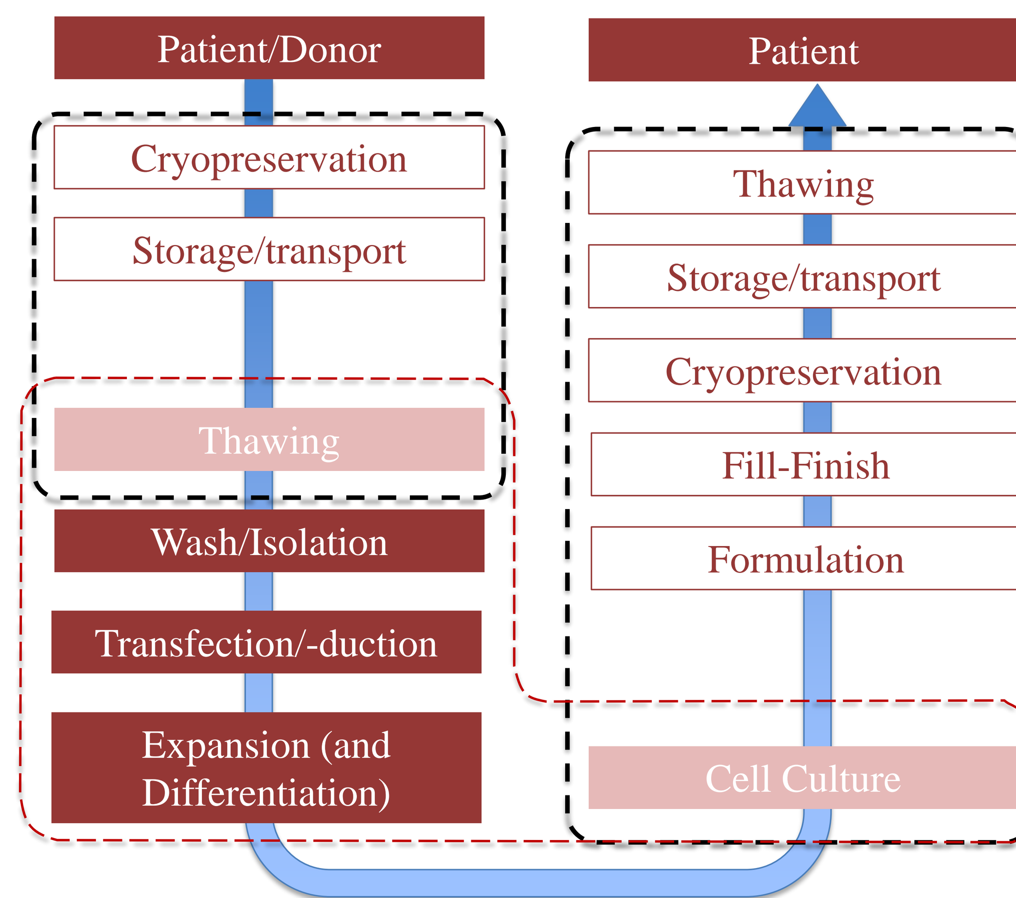
INTRODUCTION

Cell-based therapies require specialized handling to ensure their successful transport from the manufacturing facility to the patient. To provide logistical flexibility, many current commercialization models employ a frozen cell product that can be delivered to the clinic and stored, then thawed and infused into patients on demand. However, post-thaw viability and expansion of many cell types dramatically suffer from cryopreservation-induced stresses, collectively known as Delayed Onset Cell Death (DOCD). As such, it is assumed that some cell types cannot be cryopreserved and successful commercial models should be based on fresh cell culture and delivery. Cryopreservation critical process steps overlap with manufacturing process immediately prior to and after the end of the cell culture processing. As such, the efficiency and efficacy of the final therapeutic dose, as well as the manufacturing process, are directly affected by cryopreservation critical process parameters. Hence, thorough understanding of the cryopreservation impact on cells is vital for successful commercial manufacturing of cellular therapies. In this study, we investigated the impact of some of the most well-known, and also some of the more obscure, critical process parameters (CPPs) on post-thaw viability and proliferation in a Jurkat T cell model. Our results suggest that, next to incorporation of Biopreservation Best Practices for cryomedia formulation, other seemingly unimportant and irrelevant process parameters, that may generally be neglected, can have a significant impact on cell viability and proliferation post-thaw.



Cryopreservation critical process steps overlap with manufacturing process immediately prior to and after the end of the cell culture processing, therefore, directly impacting the manufacturing process as well as the efficacy and efficiency of the therapeutic dose.

METHODS

Cell Culture: The Jurkat (Clone E6-1) human acute T-cell leukemia (ATCC, VA) was cultured in complete growth medium (CGM) comprised of RPMI 1640 (Lonza, MD) supplemented with 10% v/v fetal bovine serum (FBS, Atlas Biologicals, CO).

Cryopreservation Media: Two major types of cryomedia were used in this study: (1) Home-brew mix was prepared by dissolving Human Serum albumin (HSA, Cellastim, USA) in PlasmaLyte-A at 10% w/v and supplementing with 5% v/v Dimethyl Sulfoxide (DMSO). The solution was then sterile-filtered before use; and (2) GMP-manufactured, serum-free and protein-free CryoStor CS5 (contains 5% v/v DMSO).

Cryopreservation: Jurkat T cell pellets were suspended in the indicated cryomedia and cell density, then placed in 2 ml FluidX cryovials (Brooks Life Sciences, MA), and were incubated at 2-8°C for 15 min. Cryovials were then transferred to a LN2-free controlled-rate freezer, and cryopreserved at a rate of -1°C/min. After reaching -70°C, the vials were transferred to LN_2 storage for a minimum of 24 h. Samples were thawed under the indicated conditions, and were resuspended in CGM (1:10 dilution), and transferred to the incubator at 37°C and 5% CO2.

Cryopreservation CPPs: For a set of CPPs, Standard of Practice values were compared to values representing alternative options or plausible variabilities in the process, as below:

CPP	Standard	Variation
Cryomedia	CryoStor CS5®	Home-brew (HSA in PLA and DMSO)
Feeding timeline	1-day prior to freeze	3-day prior to freeze
Cell density	5 M/mL	50 M/mL
Nucleation	Performed at -10°C	Not performed (Spontaneous nucleation)
Post-thaw rest time and temperature	0 h @ 37°C (immediate dilution and return to culture)	1 h rest, @ 2-8°C or room temperature (20-25°C), before return to culture
Dilution practice temperature	Warm media (37°C)	Cold media (2-8°C)

Viability & Expansion: Cell viability and count was evaluated based on membrane integrity immediately and at 24 h post-thaw, using Via-1 cassettes on a NucleoCounter NC-3000 imaging cytometer (ChemoMetec, Denmark).

Statistical Analysis: For all measurements, data represent the mean of 3-11 independent experiments and are normalized to pre-freeze conditions. Groups were analyzed for statistical significance via two-way ANOVA with Tukey correction and $p<0.05$ set *a priori*. Error bars represent standard deviation (SD).

RESULTS

1- Nucleation: At immediate post-thaw, no significant difference was observed in viability and recovery of the nucleated and non-nucleated samples. However, follow up analysis at 24 h post-thaw revealed a significant drop in viability and proliferation in non-nucleated samples. Such negative impact was observed regardless of the type of cryomedia (Home-brew or CryoStor CS5). Conversely, the variability (represented by SD) was larger in the HB group vs. those cryopreserved in CS5.

2- Dilution: Post-thaw dilution in cold media resulted in significant loss of viability and slower proliferation in Jurkat T cells. This may be attributed to slow DMSO removal from cells at cold temperatures, causing osmotic cell swelling and resulting in direct cell lysis or triggering the apoptotic cascade. Such negative impact on cells is minimized when diluted in warm media.

3- Post-thaw rest: Compared to the practice of immediate dilution upon thaw, resting within the cryomedia up to 1 h prior to dilution into complete growth medium has minimal impact on Jurkat T cell viability and proliferation in culture. The temperature during the rest period appeared to have no significant effect on the assay results, suggesting 1 h stability post-thaw was supported by either cryomedia.

4- Cell density: Whether cryopreserved at 5 M/mL or at 50 M/mL, no difference was observed in viability and proliferation of Jurkat T cells immediately or at 24 h post-thaw compared to cells cryopreserved using Standard CPPs. Cryomedia performance was statistically identical at high or low cell densities.

5- Cryomedia: The results suggest that, using the Standard CPPs, both CryoStor CS5 and home-brew formulation, composed of PLA, 10% w/v HSA, and 5% v/v DMSO, performed similarly in preserving viability and proliferation capacity of Jurkat T cells. However, CryoStor CS5 appeared to significantly minimize the stresses due to dilution or stochastic nucleation, as such, protecting the cells against variations in cryopreservation CPPs (or, non-standard CPPs), resulting in improved viability and count at 24 h post-thaw and decreasing variability in the results.

6- Feeding timeline: The most pronounced CPP in this study was the timing of culture feeding prior to cryopreservation. Despite appearing as a very healthy culture prior to cryopreservation (with 93% viability), the 3-day old culture appeared to be significantly more susceptible to cryopreservation-induced stresses, hence demonstrating significant loss in both viability and post-thaw proliferation.

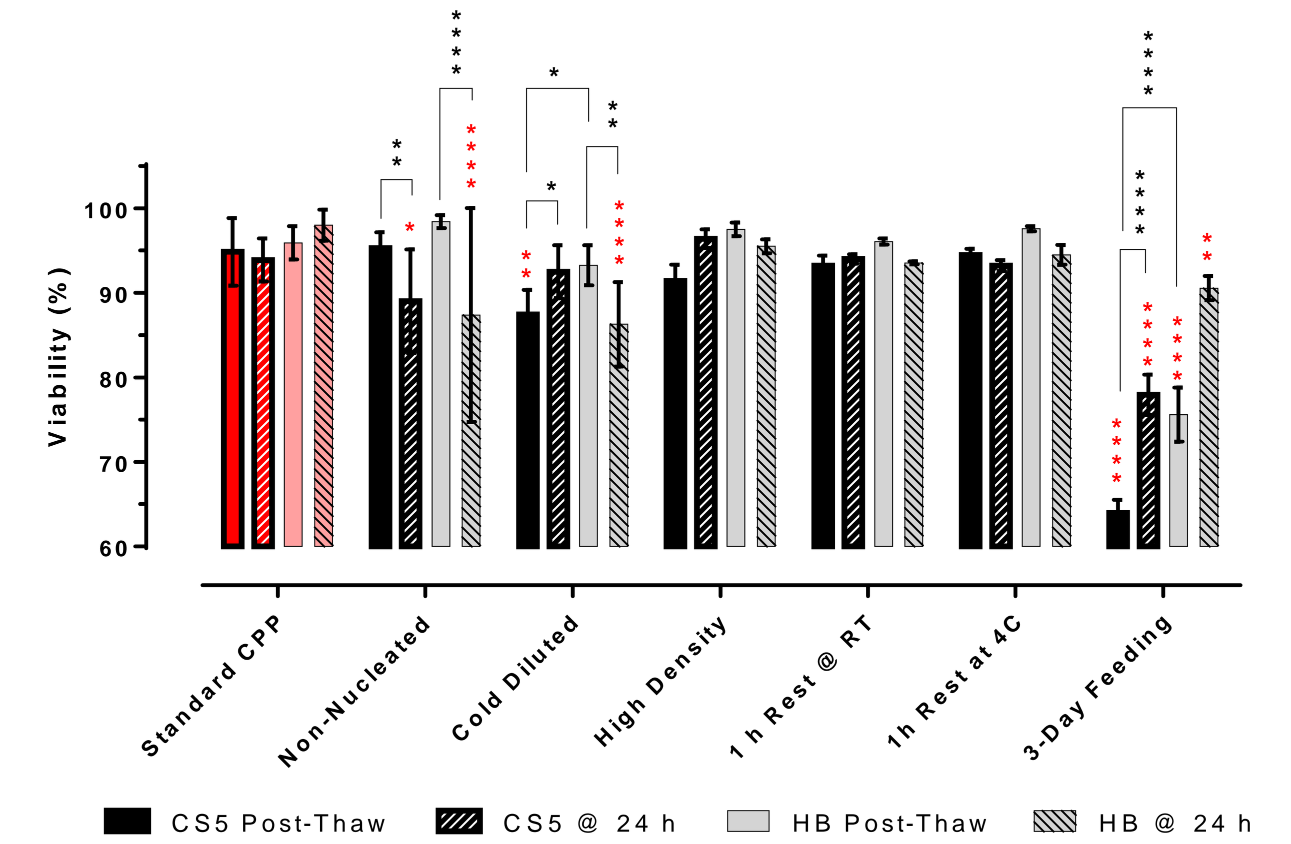


Figure 1: Viability of Jurkat T cell, assessed using a membrane integrity assay at immediately and 24h post-thaw. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$). Red asterisks (*) refer to comparison with Standard Practice results. Error bars denote SD. CS5: CryoStor® CS5; HB: Home-brew

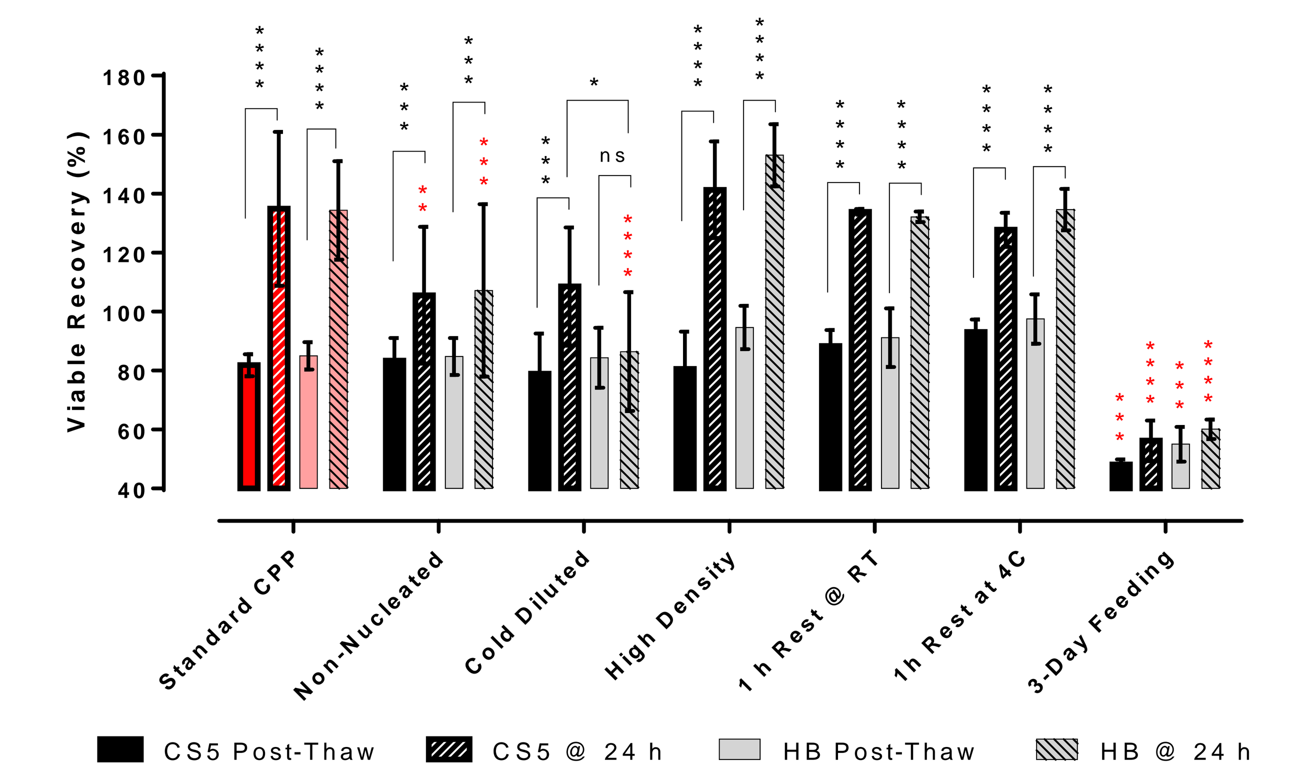


Figure 2: Viable recovery and expansion of Jurkat T cells post-thaw assessed using a membrane integrity assay. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$). Red asterisks (*) refer to comparison with Standard Practice results. Error bars denote SD. CS5: CryoStor® CS5; HB: Home-brew

CONCLUSIONS

- Immediate post-thaw analysis of viability and count is not reflective of a successful cryopreservation process. Adverse effects of non-optimized cryomedia may not be detectable until 24-48 hours post-thaw, attributed to cryopreservation-induced Delayed Onset Cell Death (DOCD). Incorporation of optimized cryopreservation media, on the other hand, can protect against DOCD; hence, resulting in decreased variability and a more accurate estimation of post-thaw survival and proliferation.
- Timing of feeding and media change prior to cryopreservation can have a significant impact on outcome of the cryopreservation process. This may be attributed to a number of parameters including accumulation of stresses that does not appear in pre-freeze viability assay, but combined with DOCS, result in significant loss of viability and function post-thaw.
- The stochastic nature of the ice nucleation inherently results in variations in cryopreservation induced stresses endured by the cells during the cryopreservation process. Hence, proper nucleation has a significant impact on viability and proliferation in model T cells.
- Dilution in 37°C media (similar to patient administration) is the optimal post-thaw dilution practice. It is highly recommended that for assaying purposes, the cells also be diluted in warm media to minimize the osmotic cell swelling and lysis during the wash process.