Optimising controlled rate freezing protocols

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Introduction

For effective delivery of many Regenerative Medicine products, a cryopreservation cold chain is essential. However, cryopreservation can be damaging to biologics if optimal cooling, storage, and thawing strategies are not used. This is further complicated by the cGMP requirements for medicinal products precluding liquid nitrogen use in clean room settings.

This study explores the key physical and biological events during the cryopreservation of Jurkat (human immune suspension cell line) and CHO (Chinese Hamster Ovary), from above 0°C to below -140°C.

Methods

Viability tests. CHO cells were cryopreserved at 10^6 cells/ml, and Jurkats at $3x10^6$ cells/ml. Both were cryopreserved at 1°C/min in 10% DMSO using a VIA Freeze controlled rate freezer (Asymptote, GEHC, Cambridge UK). At defined temperatures, sets of cells were plunged directly into liquid nitrogen to induce uncontrolled cooling, as shown in Fig 2 . Vials were stored below -140°C for at least 24h before thaw in an Asymptote SC2 vial thawer. Viable cell number was assessed 24, 48, and 72h post thaw using a Cytell imaging system.

DSC tests. DSC measurements were carried out on cell pellets or cryoprotective solutions using a power compensation DSC (Diamond, Perkin Elmer LLC, Norwalk, CT, USA) equipped with a liquid nitrogen cooling accessory (CryoFill, Perkin Elmer). The 2nd derivative heat flow was calculated between -150°C and 0°C when warming at 10°C/min, after 1°C/min cooling, with results compared to cycles containing only cryoprotective medium.

FTIR tests. Spectra in the mid-IR region from 4000 to $900cm^{-1}$ at a $4cm^{-1}$ resolution were recorded at 45 second intervals during cooling from 37°C to -50°C and warming to 60°C at rates of 2°C/min from 32 co-added scans, while continuously purging the optical bench with dry air (Balston, Haverhill, MA, USA). The position of infrared vibration bands of interest – the symmetric CH_2 stretching vibration (νCH_2) and the bend-libration H_2O combination band – from each FTIR spectrum was determined.

Results

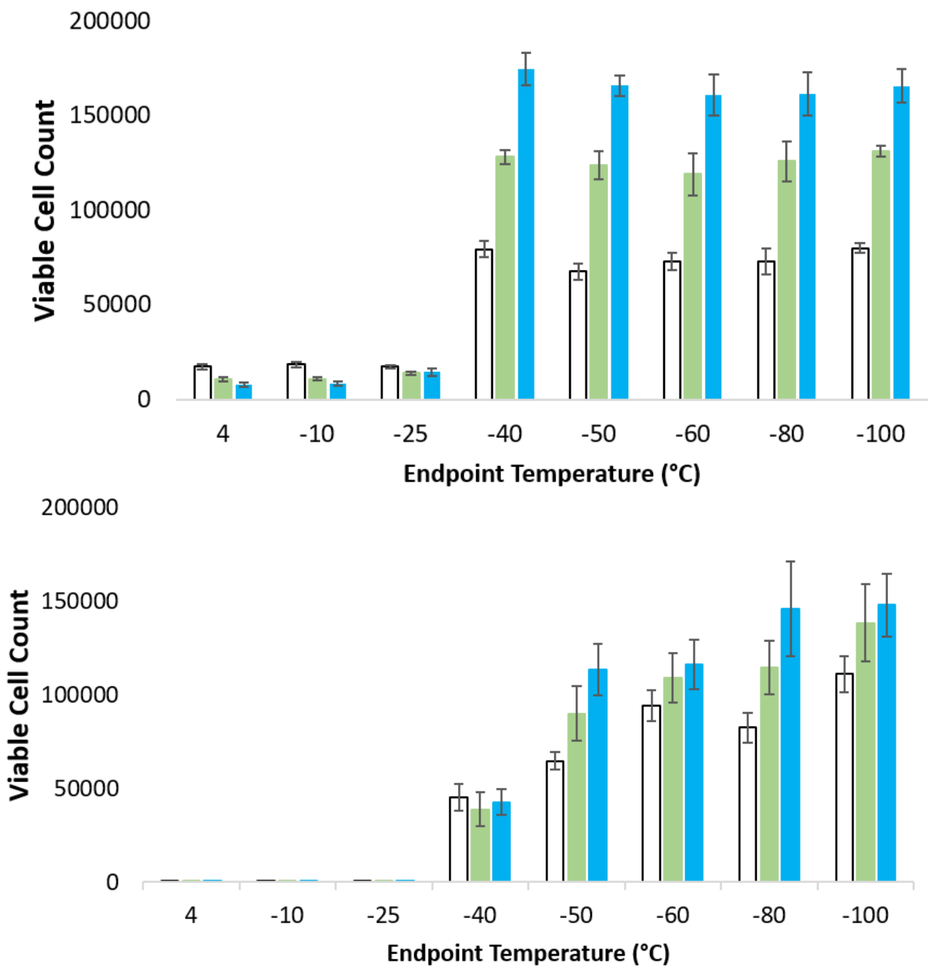

Fig 1 – Viable cell number 24h (white), 48h (green), and 72h (blue) post-thaw with different controlled cooling endpoints Data for CHO and Jurkat cells are shown top and bottom respectively. N=5 ± SD

As can be seen in Fig 1, there was no significant difference in CHO cells control cooled to only -40°C, or those cooled in a controlled manner to -100°C. Additional cooling below -40°C conferred no additional advantage to the cells post-thaw. The same held for <-50°C for Jurkat cells.

Conclusions

We have shown in this work that the key temperature during linear cooling is between -40°C and -50°C, and cooling to below this point confers no additional advantages. Physically this is due to an intracellular colloidal glass transition occurring at -47°C, after which the intracellular compartment solidifies. When this compartment solidifies the cells cannot respond osmotically and there is no free water to form intracellular ice – eliminating the key factors for cryopreservation-induced cell death.

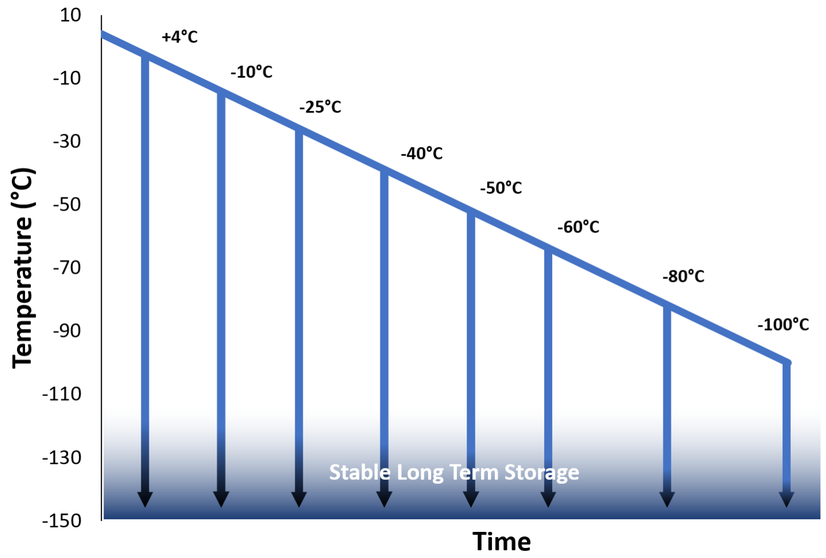

Fig 2 – A schematic of the biological controlled cooling experimental design

Fig 3 shows membrane phase transitions occur around and just above 0°C, corresponding to a transition from a liquid crystal to a gel state. As was seen in Fig 1, the critical biological temperature for the controlled cooling endpoint falls below this temperature and event.

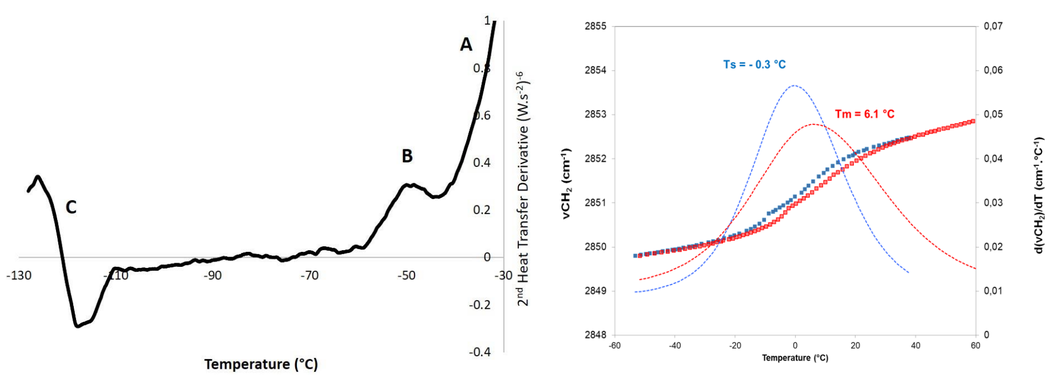

Fig 3 – Left – 2nd order derivative of a DSC trace of Jurkat cells during warming, the sample was initially cooled at 1°C/min in a solution of 10% (v/v) DMSO in culture medium to -150°C. Right - membrane phase transitions seen with FTIR during cooling (blue) and warming (red) of jurkat cells in a solution of 10% (v/v) DMSO.

Three distinct thermal events are apparent from the DSC trace in Fig 3. The gross melting of the extracellular solution (A), the intracellular glass transition (B), and the extracellular glass transition (C). Transition B is absent in a DSC trace of freezing solution without cells. The transition offset B was observed to occur at -46.7 ± 1.3°C. This corresponds to the critical temperature observed in Fig 1.

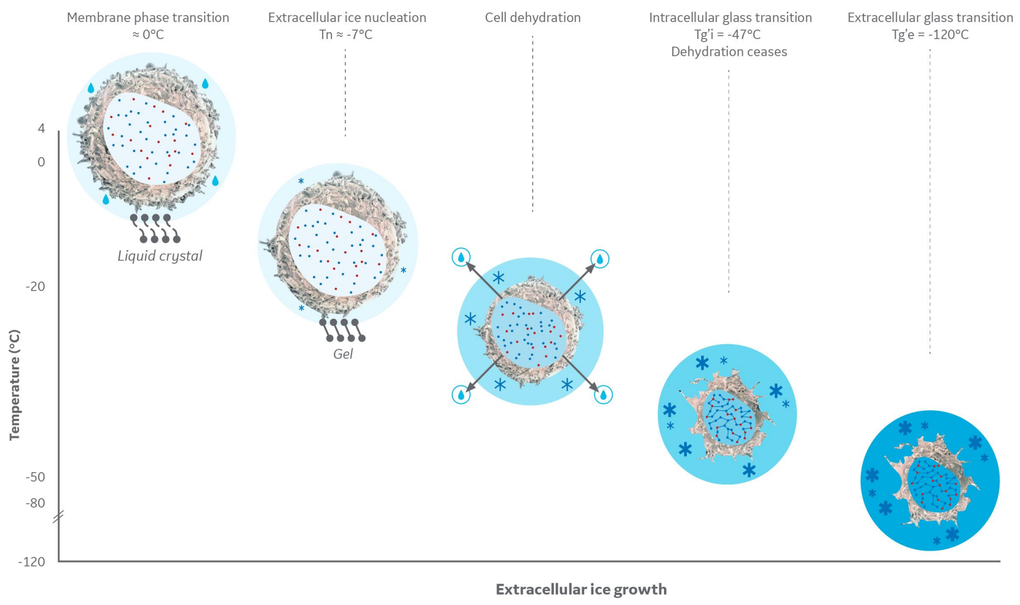

Fig 4 – Schematic of the key thermal events during cryopreservation

Discussion

Fig 4 shows the key events affecting the cell during cryopreservation. Image 1 shows the cell above 0°C in its natural state. As the cell cools in image 2, ice forms outside the cell and the membrane undergoes a gel transition. The cell starts to dehydrate to remain in equilibrium with the freeze-concentrated extra-cellular environment. Image 3 shows the cell continuing to dehydrate as pure water is locked away as ice extra-cellularly, and proteins (indicated as red and blue dots inside the cell) concentrate. Image 4 shows the cell at the intracellular glass transition temperature. Proteins cause a colloidal glass to form and the cell can safely be placed in ultra-low storage. Damage can still occur over extended periods until the extracellular component undergoes a glass transition in image 5.

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Horizon 2020 - MSCA - RISE - 2017
Research and Innovation Staff Exchange

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N° 777657