



## INTRACELLULAR ICE FORMATION IN ADIPOSE DERIVED ADULT STEM CELLS (ASC) IN THE PRESENCE OF POLYVINYLPIRROLIDONE (PVP)

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### SUMMARY

The main objective of this work was to optimize the serum free, DMSO free cryopreservation protocol for adipose derived adult stem cells (ASCs), suspended in polyvinylpyrrolidone (PVP) solution, which would help to avoid intracellular ice formation (IIF) – a well known cause of cell death during cryopreservation [1]. Passage 1 (P1) ASCs were cultured in our lab, washed and suspended in a 10% w/v solution of PVP in 1x Phosphate Buffered Saline (PBS). The freezing experiments were carried out using a fluorescence microscope (Nikon Eclipse E600) equipped with a cryostage using two different protocols – protocol 1 and protocol 2 (details in ‘materials and methods section’) at different freezing rates viz. 1, 5, 10, 20 & 40 °C/min. Occurrence of IIF was determined by observation of ‘flashing’ (sudden blackening of the cells during the freezing ramp). Protocol 1 yielded nearly 100% IIF for all the freezing rates studied whereas protocol 2 yielded 0, 40, 47, 67 & 100% IIF for the above mentioned freezing rates respectively. The results indicated that employing protocol 2 for serum free cryopreservation may yield a better post-thaw viability for P1 ASCs.

### INTRODUCTION

During freezing of cells in suspension two major phenomena that occur are cellular dehydration (loss of cell-water to the extracellular medium) and IIF [2,3]. During cooling, water in the extracellular medium freezes first, increasing the concentration of solutes in the unfrozen fraction of the medium. As a result of the osmotic pressure gradient thus created across the cell membrane between the cell cytosol and the extracellular medium, the cell starts losing water which in turn increases the concentration of the cytosol. At relatively ‘slow’ cooling rates, this process of mass transfer is governed by membrane transport properties namely the reference hydraulic conductivity ( $L_p$ ) and activation energy of hydraulic conductivity ( $E_{LP}$ ). Alternatively, with ‘faster’ cooling rates, heat transfer dominates over mass transfer and increases the likelihood of ice formation in the supercooled intracellular medium. This phenomenon depends on two nucleation parameters – a

thermodynamic parameter ( $\kappa_{SCN}$ ) and a kinetic parameter ( $\Omega_{SCN}$ ). Hence to model systems during freezing both water transportation and intracellular ice formation need to be studied. In this study we employed two different protocols to freeze the ASCs in suspension and observe the difference in percentage IIF in cells for different freezing rates.

### MATERIALS AND METHODS

Passage 1 ASCs were cultured using 6-well plates in stromal medium (DMEM high glucose, 10% fetal bovine serum, 100 units penicillin/ml 100 µg streptomycin/ml and 25 µg amphotericin/ml) for 80-90% confluence in a 5% CO<sub>2</sub>, humidified, 37 °C incubator. For cryomicroscopy experiments, the cells were trypsinized and transferred to a 1.5 ml centrifuge tube, spun down for 5 minutes at 1200 rpm and re-suspended in 10% w/v PVP solution in 1x PBS. For the experiments, 2.5 µl of the sample was transferred to a BCS 196 cryostage developed by Linkam Scientific (Surrey, UK) capable of controlled cooling and heating between -125 and 160 °C at rates between 0.1 and 130 °C/min within an error of  $\pm 1$  °C. The microscope was fitted with a ‘Photometrics Coolsnap cf’ camera (Hamamatsu, Photonics, Bridgewater, NJ). During the experiments live video signal was sent from the camera to a Dell computer and recorded.

*Freezing protocol* : Two different protocols were used to freeze the cells as shown below.

	Ramp	Rate (°C/min)	Limit (°C)	Hold (min)
<b>Protocol 1</b>	1	20	-10	-
	2	1	-13	2
	3	1, 5, 10, 20, 40	-40	-
	4	20	+20	-
<b>Protocol 2</b>	1	20	-10	-
	2	1	-13	-
	3	10	-5	-
	4	1, 5, 10, 20, 40	-40	-
	5	20	+20	-

## RESULTS AND DISCUSSION

The calculated equilibrium freezing point of 10% PVP in 1x PBS is approximately  $-0.8\text{ }^{\circ}\text{C}$  [4]. However because of cooling at finite rates it underwent extensive supercooling and froze at  $\sim -11.5\text{ }^{\circ}\text{C}$ . Nucleation at this low temperature was very rapid with growth of very fine crystals as seen from the video images (Fig 1B). During both the protocols this extracellular phase change occurred during ramp 2 and was accompanied by high percentage ( $\sim 85$  to  $100\%$ ) of IIF immediately after phase change of the extracellular medium (Fig 1B). The number of cells undergoing IIF during this ramp seemed to be largely stochastic. For protocol 1 the rest of the ramps (ramps 3 & 4) were thus largely ineffective as most of the cells were already internally frozen. In case of protocol 2, the sample was partially thawed back to  $-5\text{ }^{\circ}\text{C}$  (ramp 3) where all the intracellular ice was seen to have melted and the extracellular ice partially so. In ramp 4 ice nucleation started at a much higher temperature ( $\sim -6$  to  $-7\text{ }^{\circ}\text{C}$ ) and was much slower with noticeably larger ice crystals (Fig 1C & D), presumably because of the fact that small ice crystals from the partial thawing in the previous ramp already existed in the medium, which helped in nucleation of the extracellular medium in this ramp. For different cooling rates chosen in ramp 4 the observations were as follows : for  $1\text{ }^{\circ}\text{C}/\text{min}$ , no IIF occurred till  $-40\text{ }^{\circ}\text{C}$ . The cells underwent dehydration characterized by shrinkage in size. For  $5, 10, 20$  &  $40\text{ }^{\circ}\text{C}/\text{min}$  cooling rates,  $40, 47, 67, 100\%$  IIF was observed between  $-5$  and  $-40\text{ }^{\circ}\text{C}$ , respectively (shown in Fig 2).

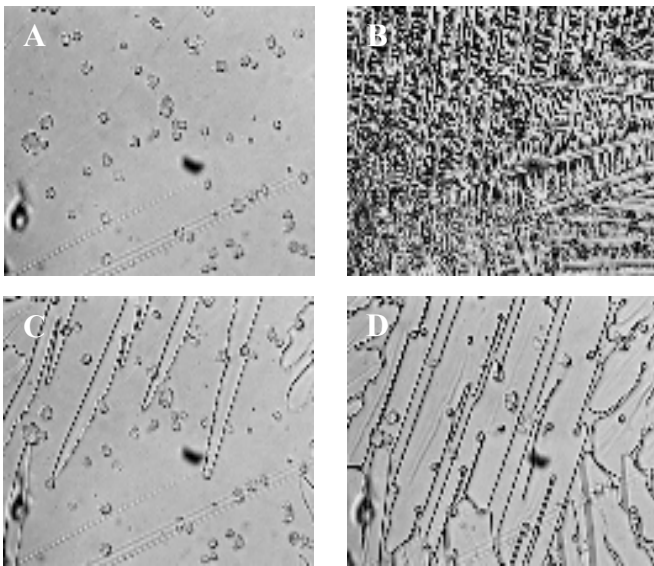


Fig 1: images taken during protocol 2 : A) Cells before start of ramp 2. B) rapid ice nucleation of the extracellular medium with fine crystals during ramp 2 and cells showing IIF. C) ice nucleation in ramp 4 with  $1\text{ }^{\circ}\text{C}/\text{min}$  cooling rate. D) cells undergoing shrinkage in ramp 4 as ice nucleation progresses

It must be noted that ramps 1 & 2 were same for both the protocols for all cooling rates chosen in ramp 4. The fact that the cells underwent IIF a second time during rapid cooling in ramp 4 (for protocol 2), after IIF in ramp 2, with

$5, 10, 20$  &  $40\text{ }^{\circ}\text{C}/\text{min}$  cooling rates, at temperatures markedly lower than phase change temperature indicates that the IIF in ramp 2 did not damage the cell membrane. A completely disintegrated cell membrane during the IIF in ramp 2 (for protocol 2) would imply that the cells would not experience any further IIF in the subsequent ramps as there would be no intact membrane to hold the cell cytosol separate from the extracellular medium. However, as IIF was observed with all cooling rates except  $1\text{ }^{\circ}\text{C}/\text{min}$  shows that  $1\text{ }^{\circ}\text{C}/\text{min}$  was a cooling rate sufficiently slow enough for the cells to undergo dehydration and avoid IIF till  $-40\text{ }^{\circ}\text{C}$ .

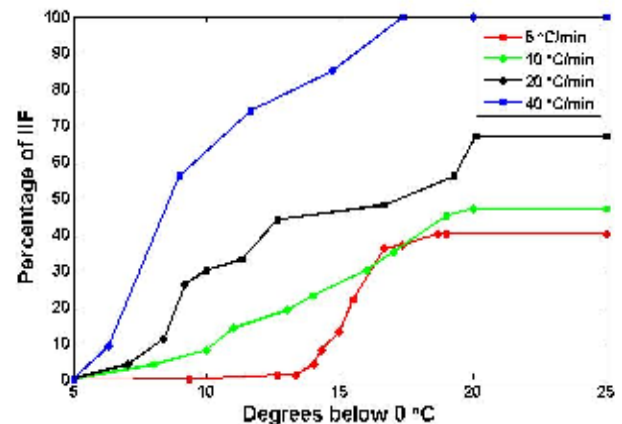


Fig 2 : Plots of temperature vs percentage of IIF (till  $-25\text{ }^{\circ}\text{C}$ ) for different cooling rates in ramp 4 for protocol 2.

## CONCLUSIONS

The findings from this study show that P1 ASCs suspended in 10% PVP solution experience lowest IIF occurrence when frozen at  $1\text{ }^{\circ}\text{C}/\text{min}$  using protocol 2 which includes a partial thawing ramp (ramp 3). It also shows that the IIF during the previous ramp (ramp 2) is not lethal enough to damage the cell membrane.

## ACKNOWLEDGEMENTS

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