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Sensitivity of human embryonic stem cells to different conditions during cryopreservation

Yanqing Xu [a], Liang Zhang [a, b], Jiandong Xu [a], Yuping Wei [a], Xia Xu [a, *]

[a] State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, PR China
[b] University of Chinese Academy of Sciences, Beijing 100049, PR China

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ABSTRACT

Low cell recovery rate of human embryonic stem cells (hESCs) resulting from cryopreservation damages leads to the difficulty in their successful commercialization of clinical applications. Hence in this study, sensitivity of human embryonic stem cells (hESCs) to different cooling rates, ice seeding and cryoprotective agent (CPA) types was compared and cell viability and recovery after cryopreservation under different cooling conditions were assessed. Both extracellular and intracellular ice formation were observed. Reactive oxidative species (ROS) accumulation of hESCs was determined. Cryopreservation of hESCs at 1 °C/min with the ice seeding and at the theoretically predicted optimal cooling rate (TPOCR) led to lower level of intracellular ROS, and prevented irregular and big ice clump formation compared with cryopreservation at 1 °C/min. This strategy further resulted in a significant increase in the hESC recovery when glycerol and 1,2-propanediol were used as the CPAs, but no increase for Me_2SO. hESCs after cryopreservation under all the tested conditions still maintained their pluripotency. Our results provide guidance for improving the hESC cryopreservation recovery through the combination of CPA type, cooling rate and ice seeding.

1. Introduction

Since human embryonic stem cells (hESCs) are capable of self-renew and differentiation into all somatic cells, they are biomedically one of the most important cell types for tissue engineering and cell therapy to treat diseases, such as diabetes and Parkinson [1]. Long-term preservation of a large amount of hESCs is required for the potentially commercial applications in regenerative medicine [2–5]. Cryopreservation is the best choice for long-term storage of cells and provides a possibility to bring hESCs from laboratories to markets at industrial scale. However, the main challenges are the low cell viability and function loss of hESCs after cryopreservation [6–9], which blocks the road to the commercialization of hESCs in therapeutic applications.

Cells are exposed to various stresses during cryopreservation, such as osmotic stress and cold stress, which could cause freezing damages to cells, and may eventually lead to cell death. Successful cryopreservation depends on the comprehensive understanding of damage effects of freezing at the cellular and membrane level. When lowering temperature during cryopreservation, ice is initially formed in the extracellular solution and solutes are pushed into the unfrozen extracellular solution, which leads to osmotic non-equilibrium across the cell membrane [10]. Cell responses to the osmotic non-equilibrium are related to the cooling rate. Slow freezing can cause cell dehydration and eventually lead to "solution effects" injury when cells are exposed to hypertonic environment for extended time period [11–14]. Also, the excessive shrinkage at slow cooling rates can cause structure damages to cells [15]. At rapid cooling rates, intracellular water does not have enough time to escape from cells, leading to the intracellular ice formation and the ice nucleation, which is thought to result in irreversible damages to cells [16–18]. Therefore, cooling rate is a critical factor affecting cell membrane integrity and ice formation during

Abbreviations: AP, alkaline phosphate; bFGF, basic fibroblast growth factor; BCIP-NBT, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium; BSA, bovine serum albumin; CPA, cryoprotective agent; DMEM/F12, Dulbecco's modified eagle's medium F12; DHE, dihydroethidium; EIF, extracellular ice formation; MEF, mouse embryo fibroblast; hESCs, human embryonic stem cells; SR, serum replacement; ROS, reactive oxidative species; TPOCR, theoretically predicted optimal cooling rate; PBS, phosphate-buffered saline; PI, propidium iodide.

* Corresponding author. No. 1 Beiertiao, Zhongguancun, Haidian District, Beijing 100190, PR China.
E-mail addresses: xuyanqing1006@hotmail.com (Y. Xu), zhangliang@ipe.ac.cn (L. Zhang), jdxu@ipe.ac.cn (J. Xu), ypwei@ipe.ac.cn (Y. Wei), xuxia@ipe.ac.cn (X. Xu).

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cryopreservation, which has a strong impact on the cell viability and recovery after cryopreservation [19].

Cell survival rate after cryopreservation is also related to cell type, cell status, CPA type and cooling methods [2,7,8,20–23]. It has been indicated that hESCs, not like mouse embryonic stem cells [3], are more sensitive to freezing damages during cryopreservation with only quite a small proportion of cells capable of forming colonies after cryopreservation [22,24,25]. Furthermore, cells in adherent status are more fragile than in suspension during cryopreservation [26]. The combination of different CPAs is useful to improve cell recovery [8,27]. Additionally, during freezing, samples experience supercooling in which spontaneous freezing of liquid is initiated at a temperature below its melting point and can be indicated by the release of latent heat of fusion [28]. Spontaneous phase change from liquid to solid at supercooling causes detrimental effects to cells during cryopreservation. Ice seeding during cryopreservation is regarded to be an efficient method to avoid supercooling effects [29]. However, the effect of ice seeding on the recovery of hESCs is rarely investigated. In this study, the responses of hESCs after cryopreservation at different cooling rates with or without the ice seeding in the presence of different CPAs were investigated.

2. Materials and methods

2.1. Cell culture

The hESCs (line H9) from WiCell Research Institute were used to evaluate cell sensitivity to different cooling conditions. hESCs were cultured at 37 °C with 5% CO_2 on gelatin (Sigma, USA) coated plates with the feeder layer of mitomycin C-inactivated mouse embryo fibroblast (MEF). The complete medium was made with 78% Dulbecco's modified eagle's medium F12 (DMEM/F12, Gibco, Life Technologies, USA), 20% KnockOut™-serum replacement (KSR, Gibco, Life Technologies, USA), 1% MEM non-essential amino acids solution (Hyclone, Thermo Scientific, USA), 2 mM L-glutamine (Sigma, USA), 0.055 mM 2-mercaptoethanol (Gibco, USA), and 10 ng/mL basic fibroblast growth factor (bFGF, Life Technologies, USA). The culture medium was changed every day. When approximately 90% confluent was reached, cells were digested with collagenase IV (Sigma, USA) at 37 °C for 25–30 min and then plated on pre-coated 6-well plates for passage, or digested using TrypLE™ Express (Gibco, Life Technologies, USA) at 37 °C for 4–5 min and collected after centrifugation at 1500 rpm for 5 min for further cryopreservation experiments. Feeder cells were removed during digestion.

2.2. Experimental solutions

To compare the sensitivity of hESCs to the cold stresses in the presence of different CPAs, three commonly used CPAs, Me_2SO, glycerol and 1,2-propanediol, were used at the concentration of 10% (v/v). The 2× CPA solutions were prepared by CPA, DMEM/F12 complete culture medium and SR at the ratio of 1:1:3, and then filtrated through 0.22 μm filter unit (Millex, Germany) before storage at −20 °C.

2.3. Cryopreservation

The dissociated hESCs were re-suspended in the DMEM/F12 complete medium, mixed with the freezing solution (2×) at the ratio of 1:1 at the density of $5–10 \times 10^4$ cells/vial (Nunc, Demark) at 4 °C for 20–30 min, frozen at the different cooling rates using a programmed freezer (Ice-Cube 14s, SY-LAB, Austria) to −80 °C, and subsequently transferred to liquid nitrogen. The cooling rate of 1 °C/min was used as a control in the presence of respective CPA. The theoretically predicted optimal cooling rates (TPOCRs) determined in our previous study (10 °C/min for Me_2SO, 25 °C/min for glycerol and 5 °C/min for 1,2-propanediol approximately) [19], 5 and 15 °C/min for Me_2SO, 10 and 30 °C/min for glycerol, and 10 °C/min for 1,2-propanediol were used to cryopreserve hESCs, respectively. The ice seeding was conducted at the sample temperature close to the freezing point of the cryoprotective medium, approximately −15 °C for glycerol and 1,2-propanediol, and −10 °C for Me_2SO. Before used for the further experiments, the cells were rapidly thawed in a water bath at 37 °C, diluted to 10 mL phosphate-buffered saline (PBS), and centrifuged to remove CPA. The cells were then re-suspended in the culture medium to the required density.

2.4. Cell viability

After cryopreservation and thawing, the cells were immediately stained with propidium iodide (PI, Fanbo Biochemicals, China) at 1 μM for 1 min in the dark. The cell viability was quantified using a flow cytometer (Cube 6, Partec, Germany). Approximately $1–2 \times 10^4$ events were analyzed for each analysis.

2.5. Cell recovery rate

After the cryopreservation under different cooling conditions, the post-thaw cells were plated into 24-well plates with feeder layer in the presence of 10 μM ROCK inhibitor Y-27632 (Selleck, USA) on the first day of culture. The cell seeding density after cryopreservation was 10^4 viable cells/well. The number of colonies was counted under an invert microscope after 10 days of the post-thaw culture. The colonies were dissociated into single cells using TrypLE Express with feeder cells removed during digestion. The cell number in each well was quantified using a flow cytometer.

2.6. Intracellular reactive oxygen species (ROS) assay

After cryopreservation, the cells were thawed in a water bath at 37 °C and diluted to PBS. CPA was removed by centrifugation. The cells were then re-suspended in PBS after PBS wash and then stained with a superoxide florescent dye, dihydroethidium (DHE, Sigma, USA) at 28 μg/mL [30] at 37 °C for 30 min in the dark. The cells were centrifuged to remove the fluorescence dye, washed with PBS to further remove the unbounded dye, and then re-suspended in PBS. The fluorescence intensity of ROS was quantified using the flow cytometer. About 1×10^4 events were measured for each analysis.

2.7. Extracellular and intracellular ice formation

A commercially available FDCS-196 cryostage system (Linkam Scientific™, UK) attached to a microscope (Nikon, Japan) was used to observe the ice formation during cryopreservation. The samples were frozen in the absence of CPAs or in the presence of 10% Me_2SO, glycerol and 1,2-propanediol, respectively, using different cooling protocols. About 1–2 μL sample was introduced into a quartz crucible with a shim spacer inside. A clean 13 × 0.17 mm glass coverslip was pressed on the top of the sample to form a thin layer which is supposed to be as thin as possible to yield better optics [31,32]. To investigate the effects of cooling rates on the ice formation during hESC cryopreservation, the sample was cooled at 1, 5, 10 and 25 °C/min in the absence of CPA. We observed how the presence of CPA affected the ice morphology when lowering the temperature at the TPOCR for different CPAs. Furthermore, due to the disadvantaged hESC survival and recovery rates when the ice

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