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Effects of osmotic and cold shock on adherent human mesenchymal stem cells during cryopreservation

Xia Xu[a,*], Yang Liu[b], Zhanfeng Cui[c], Yuping Wei[a], Liang Zhang[a]

[a] National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing, 100190, PR China
[b] Regenerative Medicine Center, Dalian Medical University First Affiliated Hospital, Dalian, PR China
[c] Institute of Biomedical Engineering, Department of Engineering Science, Oxford University, Oxford, OX1 3PJ, UK

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ABSTRACT

Cryopreservation is one of the most practical methods for the long-term storage of cell-matrix systems to ensure off-shelf availability in tissue engineering, stem cell therapy and drug testing. The aim of this study is to investigate the effects of osmotic and cold shock caused by the procedures of cryoprotectant agent addition/removal and freezing during cryopreservation on cell viability, intracellular properties, such as filamentous actin distribution, mitochondria localization and intracellular pH, and further recovery of adherent human mesenchymal stem cells. Our results shows a significant decrease in cell viability around 30% after cryopreservation at the cooling rates of 1, 5 and 10 °C/min in comparison to the adherent cells and the cells in suspension, implicating that the adherent cells are more vulnerable than the suspension cells. The osmotic shock and cold shock induced by freezing lead to dramatic changes in the intracellular properties. The cooling rate of 10 °C/min results in acidification of intracellular pH, distortion and accumulation of filamentous actin, and aggregation of mitochondria. Our findings also suggest that the cooling rate of 1 °C/min helps to maintain cell morphology and attachment, integrity and uniformity of filamentous actin, and leads to better cell recovery after cryopreservation.

1. Introduction

Long-term storage of cells and engineered tissues is important to their successful commercialization and clinical applications. Cryopreservation is a practical and effective approach to preserve cells and tissues in the frozen state for extended periods of time to ensure off-shelf availability in biomedical process and reproducible results in research. Cryopreservation of individual cells has been successfully employed to many cell types, including chondrocytes (Xu et al., 2003a,b), pancreatic cells (Tze and Tai, 1990), mouse embryonic stem cells (Kashuba Benson et al., 2008) and mesenchymal stem cells from different species (Liu et al., 2010). Microencapsulation has been reported to protect against cryopreservation damage (Dixit et al., 1993). Although cryopreservation of cells on monolayer such as keratinocytes (Pasch et al., 1999), bovine mammary cells (Talhouk et al., 1993), human skin fibroblasts (Teasdale, 1993) and rat hepatocytes (McKay et al., 2002) has been investigated, cryopreservation of engineered tissues even in a simplified form, the adherent cells (cells on matrices), still remains unsuccessful (Borel Rinkes et al., 1992; Koebe et al., 1996).

Cryopreservation usually includes four steps: cryopretectant agent (CPA) addition, freezing and keeping in liquid nitrogen, thawing and CPA removal. Currently there are two approaches for cryopreservation of cells/tissues: slow freezing at a controlled cooling rate and vitrification at a rapid cooling rate, flash freezing in liquid nitrogen. In vitrification, introduction of high concentration of CPA to a freezing solution can lift the glass transition temperature higher than the homogeneous nucleation temperature. By applying a rapid cooling rate, for an example, 2000 °C/min for oocytes, it is then feasible to vitrify the cells/tissues without ice formation. However, due to the high concentration of CPA applied in the vitrification, a stepwise method for CPA addition and removal is required to avoid toxicity and osmotic injury. Therefore currently the slow freezing method is widely used in the cryopreservation of cells in suspension due to easy handling and scale-up.

Although cryopreservation can protect cells at a low temperature for the long-term storage, stresses resulted from cryopreservation, such as osmotic and cold shock, can cause irreversible damage to cells. Due to semi-permeability of cell membranes, osmotic shock by adding CPA to cells or removing CPA from cells results in cell volume changes, leading to the damage on the integrity of cell membrane once it is beyond the threshold (Xu et al., 2003a,b). The survival rate after cryopreservation is related to the cold shock and takes a form of inverse U-shape curve (Mazur et al., 1970). At a rapid cooling rate, water inside the cells does not have

* Corresponding author. Tel.: +86 10 82544939; fax: +86 10 82544939.
E-mail address: xuxia@home.ipe.ac.cn (X. Xu).

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enough time to permeate out and is trapped into the cells. This leads to intracellular ice formation and eventually results in cell death (Madden and Pegg, 1992). Differently, at a slow cooling rate, cell damage is caused by dehydration leading to solution effect (Mazur et al., 1972). The balance between the water permeability of membrane and the probability of intracellular ice formation determines the optimal cooling rate which is different for different cell types.

Human mesenchymal stem cells (hMSCs), a good candidate in regenerative medicine (Pittenger et al., 1999), are attractive for tissue engineering, stem cell therapy and drug testing due to the capacity of self-renew and multilineage differentiation into chondrocytes (Barry et al., 2001), osteoblasts (Pittenger et al., 1999) and adipocytes (Muraglia et al., 2000) and even neural cell lineages (Deng et al., 2001). Up to date, very few reports are published on the cryopreservation of adherent hMSCs (Ji et al., 2004; Heng et al., 2006).

The present study investigates the responses of adherent hMSCs to the osmotic and cold shock during cryopreservation, including the changes in cell viability, intracellular properties and the capacity of recovery after cryopreservation. Cryopreservation of adherent hMSCs is not only convenient for high cell-based throughput and high-content screening but also helpful for matrix selection in regenerative medicine.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were purchased from the Sigma (UK) and the culture medium from Invitrogen (UK) unless otherwise stated.

2.2. Maintenance of human mesenchymal stem cells

The human mesenchymal stem cells (Lonza, UK) were cultured in mesenchymal stem cell basal medium supplemented with mesenchymal cell growth supplement (Lonza, UK), L-glutamine and 1% penicillin/streptomycin, at 37 °C in a humidified incubator with 5% CO_2. The culture medium was changed every 3 days.

2.3. hMSC culture for evaluation of the effect of cryopreservation procedures

hMSCs were cultured on coverslips. The coverslips (13 mm) were coated with 0.1% gelatine in phosphate buffer saline (PBS) at 37 °C for 30 min, followed by one wash with α-Modified Eagle Medium (αMEM). hMSCs in suspension were plated to the coverslips in 4-well plates at the seeding density of 5×10^3 cm^2, and cultured until the further evaluation.

2.4. CPA addition and CPA addition/removal without freezing

The freezing solution composed of 10% dimethylsulfoxide (DMSO) and 10% fetal bovine serum (FBS) in αMEM. For the procedure of CPA addition only, the culture medium in the wells was first completely removed and 0.5 ml of freezing solution was transferred to the culture wells. Then the cells were incubated at 4 °C for 15 min. For the procedure of CPA addition/removal, the culture medium in the wells was completely replaced by 0.5 ml of freezing solution. After 15 min of incubation at 4 °C, the freezing solution was removed, washed with the culture medium,and then the fresh culture medium was added to the culture wells at 37 °C.

2.5. Cryopreservation of hMSCs

For the cells in suspension, the cell pellet after centrifuge was resuspended in 0.5 ml freezing solution. For the adherent cells, the culture medium was completely replaced by the freezing solution at the volume of 0.5 ml/well. After incubation at 4 °C for 15 min, the cells were frozen at a cooling rate of 1, 5 or 10 °C/min (Freimark et al., 2011) using a programmable freezer (Planar, UK) until −80 °C. Then the cells were kept in liquid nitrogen or a −80 °C freezer until the further experiments. Before use, the freezing solution was replaced by the culture medium (Xu et al., 2010a,b).

2.6. Assessment of cell morphology and cell viability

Cell morphology before freezing, immediately after cryopreservation and during subsequent culture was monitored using a microscope (Nikon, Japan). Cell viability of suspended cells immediately after cryopreservation was tested using 0.4% (w/v) trypan blue in PBS. Cell viability of the adherent cells was detected using the Calcein-AM and Propidium Iodide (PI) method. The cells before and immediately after cryopreservation were directly incubated with Calcein-AM (2 μM) and PI (1 μM) for up to 15 min in dark after CPA was removed, and then washed with PBS. The slides were mounted with the SlowFade kit containing DAPI (Invitrogen, GIBCO, UK). Stained cells were visualized by fluorescence microscopy (Nikon, Japan) connected to a cooled charge-coupled device video. The live and dead cells were counted for each image (at least 9 images for each condition).

2.7. Assessment of cell metabolic activity and proliferation

To determine the number of viable cells, the alamarBlue method was performed as previously described (Liu et al., 2010). A standard curve which is correlating with the cell number and alamarBlue reduction rate was worked out. The well without cells was used as the negative control. To evaluate the effect of cryopreservation procedures on metabolic activity and proliferation, the metabolic activity of adherent hMSCs either after CPA addition/removal or after cryopreservation was normalized to the fresh cells using the alamarBlue method (Liu et al., 2010). The fresh cells were used as the positive control.

2.8. F-actin morphology, intracellular pH and mitochondria distribution

To test the possible changes in distribution and morphology of F-actin, intracellular pH (pH_i) and mitochondria distribution during cryopreservation, the cells were stained by rhodamine phalloidin, acridine orange and MitoTracker Red, respectively (Xu et al., 2010a,b). Here, the adherent fresh cells and the cells after CPA addition only, after CPA addition/removal, and after cryopreservation at the cooling rates of 1, 5 and 10 °C/min were assessed.

2.9. Statistics analysis

All assays were conducted in quadruplicate and at least three separate experiments. Data are presented as means ± standard deviation (SD) of the means for the experiments. The statistical significance was assessed using *t*-test. A probability of $p < 0.05$ was considered significant.