High concentration of synthetic serum, stepwise equilibration and slow cooling as an efficient technique for large-scale cryopreservation of human embryonic stem cells

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Objective: To develop an efficient freezing method suitable for large-scale cryopreservation of human embryonic stem cells (hESCs).
Design: Experimental study.
Setting: Research institute.
Patient(s): None.
Intervention(s): Two genetically modified hESC lines, H9-EF1-GFP and CHA-hES3-EF1-GFP, were cryopreserved in cryovials using a combination of two equilibration methods (one-step and stepwise) and two cooling vehicles (cryo-container and program-controlled freezer). After thawing, the survival and differentiation rate were compared among groups.
Main Outcome Measure(s): The hESC survival was assessed by alkaline phosphatase staining and differentiation status was determined by flow cytometry using an SSEA-4 antibody.
Result(s): In both H9-EF1-GFP and CHA-hES3-EF1-GFP cells, the survival rate was highest in the group using stepwise equilibration and program-controlled freezer, and lowest in the group using one-step equilibration and cryo-container. In the groups using cryo-container, the survival and the frequency of undifferentiated cells in both cell lines was highly improved in a stepwise equilibration compared with one-step. Thawed hESCs were positively stained with pluripotent markers SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase. The karyotypes and expression of three germ layer markers in both cell lines were not changed after freezing/thawing.
Conclusion(s): The stepwise equilibration of Knockout Serum Replacement and cryoprotectant during freezing and thawing resulted in higher survival rates by reducing osmotic damage irrespective of cooling vehicles. (Fertil Steril® 2010;93:976–85. ©2010 by American Society for Reproductive Medicine.)

Key Words: Cryopreservation, human embryonic stem cells, modified stepwise equilibration, large-scale cryopreservation, survival rate

Human embryonic stem cells (hESCs) derived from inner cell masses of blastocysts are pluripotent cells, having the capacity to self-renew and differentiate in vitro and in vivo into a wide variety of tissues exhibiting characteristics of all three germ layers (1, 2). During the last decade, hESCs have become an important resource, providing source material for cell/tissue therapy, serving as a research tool to clarify our understanding of embryonic development, and offering a useful platform for drug development. To fully exploit the remarkable potential of hESCs, techniques for handling, culturing, and cryopreserving hESCs must be improved. Although improved culturing methods have simplified one aspect of the process and made it more clinically applicable, improvements in cryopreservation efficiency have not kept pace. Cryopreservation of hESCs is inherently difficult, typically resulting in high rates of cell death from dissociation of clumps and spontaneous differentiation after freezing and thawing. Successful cryopreservation of hESCs would allow for efficient preservation of early-passage stock and facilitate worldwide distribution to researchers. It would also enable researchers to better manage their hESC experimental schedules and minimize additional labor and monetary costs.

Two cryopreservation methods are commonly used for cells and embryos: vitrification and slow freezing with rapid thawing. Of these, vitrification has come to be considered the preferred technique for the cryopreservation of hESCs since it was first reported by Reubinoff et al. in 2001 (3). Studies of hESC cryopreservation efficiencies using various vitrification procedures have reported 70%–90% survival rates using open pulled straw (OPS) methods (3, 4). However,

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vitrification has several limitations preventing its widespread use: 1) It uses high concentrations of a cryoprotectant that is toxic to cells at room temperature and may induce differentiation of hESCs; 2) it requires strict timing and a very small volume of hESCs to get good results ; 3) its success depends on the researcher's level of expertise; and 4) it introduces the possibility of contaminating hESCs with infectious agents via contact with liquid nitrogen (LN_2) if sealed containers are not used. Collectively, these attributes make vitrification largely unsuitable for use in preparing samples for distribution to other laboratories.

Compared with vitrification, cryopreservation of hESCs using the slow freezing method yields poor results, especially when cryovials are used as containers. Richards et al. (4) reported that conventional slow freezing in cryovials produced significantly lower survival rate of hESCs than vitrification in straws or OPS (8%–10% vs. 75%–88%) and that hESCs were differentiated and/or dead after conventional slow freezing and thawing. During slow freezing, a number of stresses, including osmotic stress, stress to cell junction and cell transport systems, and disruption of organelles (5, 6), can contribute to the loss of pluripotency. In 2005, Ware et al. (7) reported a 79% survival rate with no apparent increase in differentiation using a controlled-rate freezer and straws as containers, indicating that rapid recovery and high viability can be achieved using this controlled-rate cryopreservation technique. Another study reported a similar 80% survival rate and suggested that adjusting the protocol by altering the seeding temperature, cooling rate, and final temperature before plunging into LN_2 may optimize the recovery of cryopreserved hESCs (8). The factors that these researchers identified as being critical for successful freezing were an ice crystal seed, the appropriate freeze rate, and rapid thawing. However, because the scales of those studies were small and compatible cryovessels were still limited to certain types of straws, the techniques are difficult to apply to the large-scale cryopreservation of hESCs for universal use. Moreover, in those studies, differentiation rates after thawing were either not well described or were substantially higher than those observed using vitrification.

Although slow freezing procedures using program-controlled freezers are technically more reliable and reproducible, they are expensive and labor intensive. As a matter of convenience, Ha et al. (9) had introduced a simple procedure for the small-scale freezing of hESCs using a commercial freezing container and cryovials. This procedure has provided some reasonable survival rates (10%–30%) without using a program-controlled freezer when 5% dimethylsulfoxide (DMSO) and 10% ethylene glycol in 50% fetal bovine serum (FBS) was used as cryoprotectant. Also, the cryovials they used can be sealed tightly and are capable of storing a large number of cells. Once the conditions for slow freezing using cryovials have been optimized, this procedure would be useful for long-term storage and worldwide distribution of hESCs. Even though many merits were provided, some concerns remained for general or clinical application. First of all, serum used for that study had a risk of potential pathogen contamination and variability depending on the batch. Also, survival and differentiation rates of frozen/thawed hESCs were unstable and variable depending on the level of researcher's expertise, because hESCs clumps easily dispersed into pieces during equilibration and freezing/thawing. Therefore, in the present study, we sought to develop an efficient and stable cryopreservation method for large-scale cryopreservation of hESCs using slow cooling and cryovials. For this, we have focused on a synthetic serum substitute for replacing animal serum and stepwise equilibration for reducing mechanical and osmotic damages. In fact, Thonon et al. (10) reported that a stepwise procedure for cryoprotectant (CP) equilibration improves the survival rate after thawing of in vitro–produced bovine embryos. We evaluated the effects of equilibration methods (one-step vs. stepwise) and freezing vehicles (program-controlled freezer vs. cryocontainer) on viability and spontaneous differentiation of hESCs when we used a high concentration (90%) of Knockout Serum Replacement (SR; 90%) and cryovial for hESC cryopreservation. Our results show that the most effective freezing method was a stepwise equilibration method using a program-controlled freezer for both genetically modified H9 and CHA-hES3 cell lines. More than our expectation, the survival rate of a stepwise equilibration method using a cryocontainer was significantly higher than that of a one-step equilibration method using a program-controlled freezer. Therefore, we believe that our stepwise equilibration method has a positive effect on hESC survival by reducing osmotic damage during freezing and thawing. Our slow freezing method using stepwise equilibration of SR and CP can cryopreserve a large amount of hESCs at once by enzymatic dissociation and use of cryovials as a container for both program-controlled freezer and cryocontainer. For that reason, our slow freezing method can be easily adapted for use in large-scale cryopreservation of hESCs in any laboratory and is ideally suited for good manufacturing practice laboratories and hESC banks.

MATERIALS AND METHODS

hESC Culture

This study was undertaken under approval of the Institutional Review Board (IRB) of CHA General Hospital, Seoul, Korea, and the national IRB regarding embryo research (no. 13) using human embryonic stem cells. The hESC culture medium consisted of 80% (v/v) DMEM/F12 (without pyruvate; Gibco-BRL, Franklin Lakes, NJ), 20% (v/v) SR (Gibco-BR), 100 U/mL penicillin G, 100 μg/mL streptomycin (Gibco-BRL), 0.1 mmol/L β-mercaptoethanol (Gibco-BRL), 1% (v/v) nonessential amino acid stock (Gibco-BRL), and 4 ng/mL basic fibroblast growth factor (bFGF; Invitrogen, Carlsbad, CA). The hESCs were cultured in 6-well culture plates (Nalgene; Nalge Nunc International, Rochester, NY) at 37°C in a 5% humidified CO_2 incubator. The hESCs were subcultured every 6 days by mild enzymatic dissociation with 10 mg/mL dispase (Gibco-BRL) for