Comparison of Different Vehicles on Human Embryonic Stem Cells using Vitrification

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ABSTRACT

Vitrification has been suggested to be an effective method for the cryopreservation of human ES cells. However, the efficiency of vitrification with different vehicles remains a matter of ongoing controversy. The objective of this study was to assess the efficiency of cryopreservation in human ES cells by vitrification using different vehicles. A human ES cell line and a variety of vehicles, including micro-droplet (MD), open-pulled straw (OPS) and electron microscopic grid (EM-grid), were employed in an attempt to assess vitrification efficiency. In order to evaluate the survivability and the undifferentiated state of the post-vitrified human ES cells, we conducted alkaline phosphatase staining and characterization via both RT-PCR and immunofluorescence assays. The survival rates of the post-vitrified human ES cells using MD, OPS and EM-grid were determined to be 61.5%, 66.6% and 53.8%, respectively. There also exist significant differences between slow-freezing and vitrification ($p<0.01$). However, no significant differences were detected between the vehicle types. Finally, the pluripotency of human ES cells after thawing was verified by teratoma formation. Cryopreservation using vitrification is more effective than slow-freezing, and the efficiency of vehicles proved effective with regard to the preservation of human ES cells.

(Key words : Human embryonic stem cells, Micro-droplet, Vitrification, Cryopreservation)

INTRODUCTION

Human embryonic stem (ES) cells are pluripotent cells, with the ability to self-renew and to differentiate into a wide variety of tissues which exhibits the characteristics of all three germ layers, both *in vitro* and *in vivo* (Thomson *et al.,* 1998). Since Thomson and colleagues initially described the derivation of human ES cell lines from human blastocysts in 1998, a host of human ES cell lines have been established (Thomson *et al.,* 1998; Reubinoff *et al.,* 2000; Gearhart, 1998). The newly-established human ES cells have allowed for new ground to be broken in the fields of developmental biology and transplantation medicine (Heng *et al.,* 2004). Currently, however, several obstacles must be overcome due to difficulty in the maintenance of undifferentiated characteristics *in vitro,* differentiation into target cell types using a variety of differentiating factors, and the development of efficient methods of cryopreservation in ES cell research. The enhancement of currently used cryopreservation technologies may prove particularly vital for the establishment of human ES cell banks (Gearhart, 1998). As many researchers have already reported that the characteristics of human ES cells may change during long-term culture (Buzzard *et al.,* 2004; Draper *et al.,* 2004; Mitalipova *et al.,* 2005), the relevance of maintenance and the preservation of early passage human ES cells has become a salient issue. Nevertheless, the unique biological features of ES cells limit the effectiveness of current cryopreservation methods.

Among the methods currently employed for the cryopreservation of human ES cells, the conventional slow-rate freezing method is most commonly used for the cryopreservation of a variety of cell lines, including murine ES cell lines (Robertson, 1997). However, the Reubinoff group and other scientists have reported that conventional slow-rate freezing cryopreservation and subsequent thawing result in low survival rates in human ES cells (Reubinoff *et al.,* 2001; Richards *et al.,* 2004; Zhou *et al.,* 2004; Richards *et al.,* 2004; Suemori *et al.,* 2006). Recently, the vitrification technique has been developed, and has evidenced superior efficiency and survivability in the fertilized eggs and blastocysts of mammalian species (Rall *et al.,* 1985; Karlsson, 2002). In human ES cells, vitrification manifested augmented efficiency as compared to

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conventional slow-rate freezing (Reubinoff *et al.,* 2001; Richards *et al.,* 2004; Zhou *et al.,* 2004; Richards *et al.,* 2004; Suemori *et al.,* 2006). Due to a variety of factors that might affect the outcomes of cryopreservation, including the types of vehicles employed and the cryoprotectant concentration, the optimal protocol for vitrification-mediated cryopreservation remains to be definitively determined (Liebermann *et al.,* 2002). The vehicles most frequently utilized for the cryopreservation of human ES cells include micro-droplet (MD), open-pulled straw (OPS), and electron microscopic copper grid (EM-grid) vehicles. Since Papis *et al.* (2000). initially described the successful use of the micro-droplet (MD) vehicle, this technique has been regularly utilized for the cryopreservation of bovine and porcine eggs and embryos (Papis *et al.,* 2000; Misumi *et al.,* 2003). As the MD procedure does not require the use of vehicles, it is simpler and advantageous in comparison to vehicle-based vitrification methods utilizing OPS and EM-grid vehicles (Misumi *et al.,* 2003). Thus, the strengths and weaknesses of various vehicles, which have yet be studied using ES cells, must be considered and analyzed further in order to determine the most efficient method for the cryopreservation of human ES cells.

Hence, the objective of this study was to evaluate the efficiencies of different vehicles in cryopreservation by vitrification, and to establish an optimal vitrification strategy for human ES cells.

MATERIALS AND METHODS

Culture of Human ES Cells

Human ES cell lines (CHA-hES3, passage 88~92) were utilized to evaluate the efficiency of the vitrification of human ES cells. The maintenance of these cell lines has been previously described (Ahn *et al.,* 2006). In brief, Human ES cells were grown on a feeder layer of mitomycin C-inactivated STO cells in DMEM/F12 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 20% serum replacement (SR; Gibco), basic ES medium components, 1 mM L-glutamine (Gibco), 1% nonessential amino acids (Gibco), 100 mM -mercaptoethanol (Gibco), and 4 ng / mL bFGF (Sigma, St. Louis, MO, USA). The human ES cells were mechanically isolated under a dissecting microscope, and the harvested clumps were aggregated for cryopreservation. In order to minimize damage to the human ES cells induced by freezing and thawing, the human ES cells were aggregated via 24 hr of incubation in a suspension culture in feeder on human ES cell culture medium.

Slow Freezing Method

Slow freezing was conducted via conventional slow-freezing (Freshney, 2000). Approximately 50 clumps of aggregated human ES cells were placed in vials and soaked in 1.2 ml chilling medium containing 90% fetal bovine serum and 10% dimethylsulphoxide (DMSO; Sigma). The vials were then slowly cooled (~1℃/min) in a freezing container (Nunc; Naperville, IL, USA,) to −80 ℃, and stored in liquid nitrogen. The frozen cells were then rapidly melted in a water bath at 37℃. The thawed ES cell clumps were then washed in HEPES-buffered ES cell culture medium, and plated on a fresh feeder layer.

Vitrification Method

The vitrification method used in this study was modified from the method pioneered by the Hong group (Hong *et al.,* 1999). Human ES cells were pre-equilibrated for 2.5 min in DMEM F-12 (with HEPES buffer, Gibco) supplemented with 1.5 M ethylene glycol (EG; Sigma), and 10% (v/v) fetal bovine serum (FBS; Hyclone, USA) at 37℃. These pre-equilibrated ES cells were then placed in the same volume of DMEM F-12 (with HEPES buffer) supplemented with 5.5 M ethylene glycol, 1.0 M sucrose, and 10% FBS, for 20 sec for final equilibration. After the clumps had been loaded onto different vehicles, the vehicles were immediately soaked and stored in liquid nitrogen (Table 1). The thawed vehicles were then sequentially transferred to new culture dishes containing 2 ml of DMEM F-12 (with HEPES buffer) with serial diluted sucrose (1.0, 0.5, 0.25, 0.125 M) and 10% (v/v) of FBS at 2.5 min intervals at 37℃.

To investigate characterization of post-thawing human ES cells, human ES cells were used after three passages.

Assessment of the Survivability of Human ES Cells and Spontaneous Differentiation after Cryopreservation

In order to evaluate the recovery and survivability of human ES cells after cryopreservation, we counted the clumps of post-thawing human ES cells, then assessed the degree to which the post-thawing human ES clumps expanded. The rate of recovery for the post-thaw human ES cells was calculated via comparisons of the numbers of post-thaw human ES clumps with those of the frozen human ES clumps. The survival rate was also calculated by the number of growing human ES clumps in 50 clumps after thawing. Differentiation was scored via AP staining after inspection. Basically, the classifications of post-thaw colonies for differentiation were divided into three groups, based on the observed levels of differentiation. Each of the colonies was scored as completely (>95%) undifferentiated colony (3 points as undifferentiated state), predominantly (>60%) undifferentiated colony (2 points as partial differentiated state), and predominantly (<40%) differentiated colony (1 point meaning differentiated state). According to this formula, the differentiation scores were calculated, as shown below;

Table 1. Comparison of recovery, survival and differentiation score after thawing between conventional and vitrification methods in human ES cells

Croypreservation		Clumps	Volum (μl)	Recovery rate (%)[1]	Survival rate (%)[2]	Differentiation Scorring[3]
Conventional	Slow Freezing	50	500	96.6	0	ND*
Vitrification	MD	50	8~10	87.6	61.5	174.4
	OPS	50	10	93.6	66.6	185.6
	EM-grid	50	ND	89.6	53.8	181.5

*ND: not detectable
1 and 2: the percentage of recovery and survival for human ES clumps after thawing in 50 clumps.
3: The scoring of differentiation was calculated according to the grade of differentiation.
Scoring= undifferentiation × 3 + patial differentiation × 2 + differentiation × 0

Differentiation score = undifferentiation (%) × 2 + partialdifferentiation (%) × 1 + differentiation (%) × 0

RNA Extraction and Reverse Transcriptase-PCR

Reverse transcriptase-PCR was utilized in order to identify the stemness of the human ES cells. In brief, total RNA was extracted with Trizol (Gibco), in accordance with the manufacturer's instructions. One microgram of total RNA-treated DNase (Gibco) was employed for cDNA synthesis (Superscript™ First-strand synthesis system, GibcoBRL, Life Technologies). The reverse transcription reaction was conducted in a 20 μl mixture (1×RT buffer, 1.25 mM $MgCl_2$, 5 mM DTT, 2.5 g random hexamer, 0.5 mM each of dATP, dCTP, dGTP, and dTTP, and 50 units of Superscript II enzyme) for 2 hr at 42℃. After the reverse transcription reaction, RNA was degraded by *E. coli* RNase H (2 units). PCR was conducted in 50 μl of reaction buffer containing 2 U of Takara Taq, 1×PCR buffer, 0.8 mM dNTP mixture, and 100 pmol of specific primers. Standard PCR conditions were as follows; 3 min at 95℃; followed by cycles of 45 sec of denaturation at 95℃, 45 sec of annealing at 55℃, and a 1-minute extension at 72℃. The primer sequences and expected sizes of the amplified products employed were as follows: OCT4: product 250 bp 5`-CGT GAA GCT GGA GAA GGA GA -3`, 5`-CAA GGG CCG CAG CTT ACA CA-3`; Nanog: product 255 bp 5`- GCT TGC CTT GCT TTG AAG CA -3`, 5'-TTC TTG ACT GGG ACC TTG TC-3'; GAPDH; product 287 bp 5`- CTT TTA ACT CTG GTA AAG TGG-3`, 5`-TTT TGG CTC CCC CCT GCA AAT-3`. The PCR products were verified on 1.2% agarose gel and visualized under UV light following ethidium bromide staining.

Immunocytochemistry and Histology

After several passages subsequent to thawing, human ES cells were fixed in 4% formaldehyde for 15 min and permeabilized with 0.1% Triton X-100/PBS for 10 min at room temperature. After 30 min of treatment with 5% normal goat serum at room temperature, the cells were incubated for 24 hr with mouse anti-human antibodies for Oct-4 (SC-5279,1:100, Santacruze Biotechnology Inc., CA, USA), SSEA-1 (1:100, Chemicon ; International Inc., CA, USA), SSEA-4 (1:100, Chemicon), and Tra-1-60 (1:100, Chemicon), Tra-1-81 (1:100, Chemicon) at 4℃. After washing, the bound primary antibodies were detected using FITC-conjugated goat anti-mouse IgG (1:800, Molecular Probes Inc., Eugene, OR, USA) and Rhodamine-conjugated goat anti-mouse IgG (1:800, Molecular Probes Inc) for 1 hour at room temperature. The stained slides were then washed and mounted using Vectashield mounting solution with DAPI (Vector Laboratories, Burlingame, CA, USA). In order to analyze the expression of alkaline phosphatase (AP), the cells were fixed for 20 min in 4% paraformaldehyde, and detected using an ES cell characterization kit (Chemicon) in accordance with the manufacturer's instructions. The images were then analyzed using a confocal laser scanning system (Zeiss LSM 510 META, Carl Zeiss, Jena, Germany) and an inverted microscopy system (ECLIPSE TE2000 and DXM1200C, Nikon, Kanagawa, Japan).

Karyotype Aanalysis

Chromosomal analyses were conducted in accordance with the standard protocols, with some minor modifications (Dutrillaux *et al.,* 1981). Post-thaw human ES cells were treated with 100 mg/ml of colcemid solution (Gibco) for 2 hr at 37℃, and trypsinized with trypsin-EDTA solution (Gibco). The harvested cells were then resuspended and incubated with hypertonic solution (1% citrate buffer) for 30 min at 37℃. After treatment with hypertonic solution, the cells were fixed in Carnoy's solution (methanol : glacial acetic acid = 3 : 1) three times, then dropped onto chilled glass slides. Karyotyping was conducted with G-banding for chromosomal identification. At least 20 metaphase spreads were analyzed for chromosomal rearrangements by a qualified cytogeneticist.

Teratoma Formation in NOD/SCID Mice

Clumps of undifferentiated thawed human ES cells (~50 aggregated human ESs) were injected into the testicular capsules of 8-week-old NOD/SCID mice (The Jackson Laboratory, Maine, USA). The NOD/SCID mice were sacrificed 12 weeks afterward, and the teratomas were removed. The teratomas were then frozen and serially sectioned (8 μm) using a cryostat (Leica Microsystems, Wetzlar, Germany). In order to analyze the three germ layers in the teratoma tissues, the sectioned sample slides were fixed for 20 min with 4% paraformaldehyde, and were examined histologically using Hematoxylin and Eosin stain, as well as special stains. In order to confirm the origins of the cells in the teratomas, the slides were fixed for 20 min with 4% paraformaldehyde and permeabilized for 10 min with 0.1% Triton X-100 in PBS. After 30 min of treatment with 5% normal goat serum at room temperature, the slides were incubated for 30 min with mouse anti-human nuclei (1:100, Chemicon) antibodies. Rhodamine-conjugated goat anti-mouse Ig (1:500, Molecular Probes Inc.) was used as a secondary antibody and Vectashield mounting solution with DAPI (Vector Laboratories) was used for mounting. Images were analyzed using a confocal laser scanning system (Zeiss LSM 510 META, Carl Zeiss, and Jena, Germany) coupled with an inverted microscopy system (Nikon).

Statistical Analysis

Results of the recovery rates, survival rates, and differentiation scores are expressed as means ± standard deviation (n=5 experiments). Data analysis was conducted using the Statistical Package for Social Sciences (SPSS) 10.0 statistical package. A value of $p<0.05$ was considered to be significantly different (SPSS 10.0, CA, USA).

RESULTS

Vitrification using Different Vehicles on Human ES Cells

In order to compare the effects of different vehicles on the survivability of human ES cells during vitrification, the OPS and EM-grid including micro-droplet methods were employed (Fig. 1A-C). Human ES cells could be successfully attached and recovered after thawing, via both slow freezing and vitrification. The measured recovery rates were as follows: 95.6%(±5.8%) for slow freezing, 87.6%(±3.8%) for MD, 93.6%(±2.6%) for OPS, 89.6% (±2.6%) for EM-grid, and 96.6%(±5.8%) for the positive control, which did not freeze, respectively (Fig. 1D). The positive control was employed in order to determine whether or not the obtained results had been affected by the toxicity of the cryopreservation solution itself, regardless of freezing. However, no significant differences

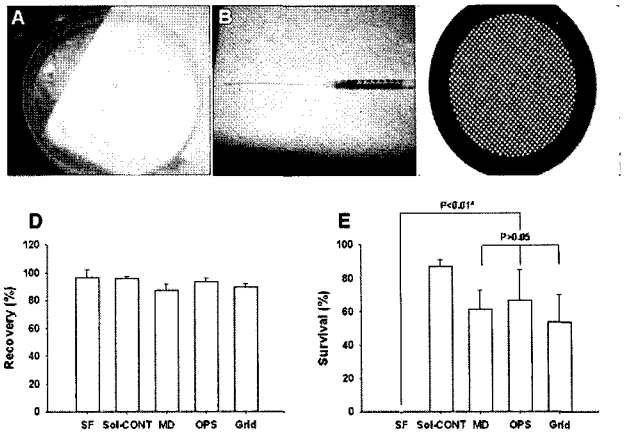

Fig. 1. Various vehicles and comparisons of efficiency for recovery and survival rate of human ES cells after thawing, via slow freezing and vitrification methods. Micro-droplet (A), Open pulled straw (B), EM-grid (C), Recovery rates (D), Survival rates (E). Significant differences were detected between freezing and vitrification using MD, OPS, and EM-grid ($p<0.01$). However, no significant differences were determined to exist between the vehicle types. These results are expressed as the means± standard deviation (SD) (n=5). A value of $p<0.05$ was considered significant.

in the recovery rate were detected among the different vehicle ($p>0.05$) groups studied. The survival rates were analyzed after seeding the clumps onto new feeders, then cultured for 6 days. The survival rate was almost 0% for SF, 61.5% (±11.5%) for MD, 66.6%(±18.3%) for OPS, 53.8%(±16.1%) for EM-grid, and 87.1%(±3.8%) for the controls (Fig. 1E). The survival rates of human ES cells using the slow freezing method were quite low, compared to what was seen using vitrification ($p<0.01$). The survival rates associated with each of the vehicle types did not differ to a statistically significant degree, whereas the rate between slow freezing and vitrification was observed to be significantly different ($p>0.05$). The results indicated that post-thawed human ES cells using different vehicles recovered almost fully, with no observed loss.

In order to evaluate the effects of cryopreservation on the level of differentiation in human ES cells, we conducted AP staining, which allowed us to detect undifferentiated human ES cells. As described in the Materials and Methods section, the grade of differentiation was classified as undifferentiated (Fig. 2A), partially differentiated (Fig. 2B), and differentiated (Fig. 2C), according to the percentage of the stained region of the clump in the post-thawed human ES cells as the result of AP staining. The differentiation scores for MD, OPS, EM-grid, and the positive control are as follows: 174.4 (±10.2), 185.6(±4.6), 181.5(±9.0), and 188.3(±6.4), respectively (Fig. 2D). Among the different vehicles, the differentiation score for OPS evidenced a greater upward trend when compared to that of other vehicles. However, no statisti-

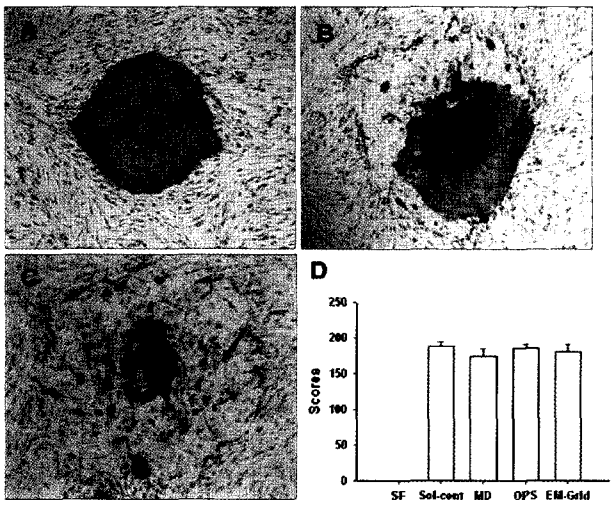

Fig. 2. The undifferentiation criteria of human ES cells after thawing was evaluated via AP staining. Undifferentiated state (A), Partially differentiated state (B, arrow; undifferentiated), Differentiated state (C, arrow; differentiated). Undifferentiational scoring of human ES cells (D). No individual differences were detected between vehicle groups.

cally significant differences were detected between the scores of each vehicle ($p>0.05$) and OPS.

Human ES Cells Sustained Self-renewal and Pluripotency *In Vitro* after Vitrification

In order to verify the self-renewal and pluripotency of post-thaw human ES cells, the cells were aggregated, attached to fresh feeder cells, and maintained using the same method as was employed for the cultivation of human ES cells (Fig. 3A-C). In order to assess the characteristics of post-thaw human ES cells, we verified the expression of a variety of undifferentiation markers, including AP, Oct-4, SSEA-1, -4, TRA-1-60, and TRA-1-81, via histological and immunocytochemical techniques (Fig. 3D-I). Karyotyping in post-thaw human ES cells was found to be normal, as 46, XY (Fig. 3J). Neither chromosomal rearrangements nor any other abnormalities were observed at any passage after thawing. In addition, the expressions of Oct-4 and Nanog, both of which are markers of the stemness of human ES cells, were detected via RT-PCR in the post-thaw human ES cells (Fig. 3 K).

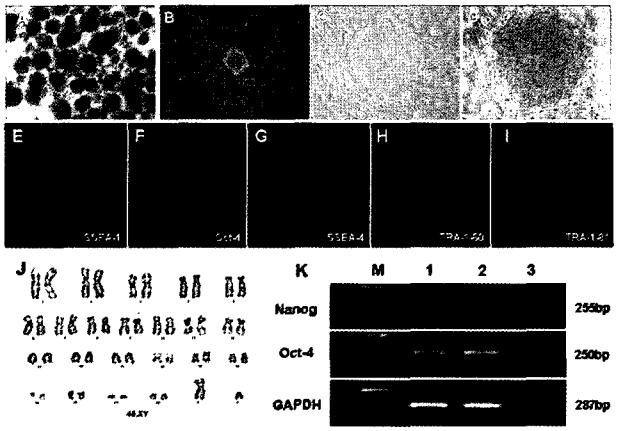

Fig. 3. Human ES cells retained self-renewal and pluripotency *in vitro* after vitrification. Human ES cells aggregated in suspension culture after 24 hours (A, ×100), Post-thawed human ES cells on fresh feeder layer (B). Attachment and growth of human ES cells (C). Alkaline phosphatase staining (D), Immunofluorescence of SSEA-1 (E), Oct-4 (F), SSEA-4 (G), TRA-1-60 (H) TRA-1-81 (I).(C~I; Magnification, ×200). Karyotype analysis of post-thawed human ES cells (J), RT-PCR of Oct-4 and Nanog expression (K): M, 100 bp DNA ladder marker; lane 1, Naïve human ES cells; lane 2, Post-thawed human ES cells; lane 3, negative control.

Human ES Cells Differentiate Three Germ Layers from Teratoma *In Vivo* after Vitrification

In order to further confirm the retention of the pluripotency of post-thawed human ES during *in vivo* differentiation, we engrafted clumps of thawed human ES cells (~50 clumps) into the testicular capsules of NOD-SCID mice, and sacrificed the mice after 15 weeks. Whole teratoma (<1.5 cm) were obtained, in which the three differentiated germ layers could be visualized via H & E staining (Fig. 4A, B). The expression of human nuclei derived from human ES cells was observed via immunofluorescence with anti-human nuclei antibodies (Fig. 4C). In addition, the results of histological analyses showed tissues representing all three germ layers in the portions of the testicular capsules extracted from mice, which were observed via Masson's trichrome staining for secretion epithelium and muscle fibers (ectoderm, mesoderm) (Fig. 4D, E), and epithelium gut-like cells (endoderm) (Fig. 4F). These data indicated that post-thawed human ES cells retained their pluripotency, and this was

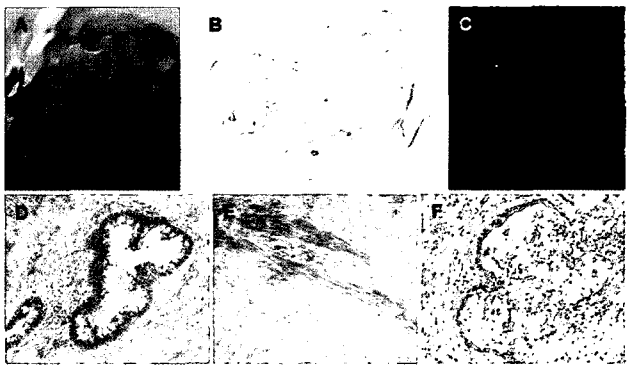

Fig. 4. Teratoma formation in human ES cells *in vivo* after vitrification. Teratoma extracted from sacrificed mice (A), Montage image of whole teratoma by H & E staining (B), Immunofluorescence of anti-human-nuclei antibody (C), special stain for the identification of three germ layers; epithelium cells (Ectoderm, D), muscle fiber cells (Mesoderm; E), Epithelium gut-like cells (endoderm; F). (C-F; Magnification, ×200).

maintained during differentiation *in vivo* with no change in the characteristics of the human ES cells following vitrification.

DISCUSSION

The present study evaluated the efficiency of the vitrification of human ES cells utilizing a variety of vehicle types, including OPS, EM-grid, and micro-droplet vehicle techniques. We also conducted an analysis of the survivability and differentiation rates, as well as an assessment of the human ES cells after thawing.

In general, the most frequently-employed techniques for the cryopreservation of human ES cells are slow-rate freezing (Ha *et al.*, 2005;, Wu *et al.*, 2005), and vitrification (Reubinoff *et al.*, 2001; Richards *et al.*, 2004; Zhou *et al.*, 2004; Richards *et al.*, 2004). Slow-rate freezing represents a modification of the traditional slow-freezing procedure employed for the storage of surplus products such as eggs, sperms, and embryos in clinical applications. However, this technique necessitates intensive labor, as well as an expensive programmable freezer, with which most laboratories are not equipped. Therefore, new cryopreservation technology is clearly necessary in order to enhance the survivability and maintenance of the characteristics of human ES cells after thawing (Ha *et al.*, 2005; Ware *et al.*, 2005; Katkov *et al.*, 2006). Thus, Reubinoff and colleagues have developed vitrification as a method for the preservation of mechanically isolated human ES cells (Reubinoff *et al.*, 2001; Richards *et al.*, 2004; Zhou *et al.*, 2004; Richards *et al.*, 2004). However, these human ES clumps dispersed readily during cryopreservation, and evidenced relatively low survival rates after thawing (unpublished data). Also, we confirmed that aggregated human ES cells suspended on feeder cells 1 day after mechanical isolation maintained their aggregated morphology well. This enhancement might be attributed to an increase in the aggregation of human ES clumps throughout the suspension culture. We believe these are essential steps in the preservation of human ES cells, with regard to the enhancement of the survival rate after thawing. These results are similar to previously reported data (Zhou *et al.*, 2004).

As Reubinoff's group reported the efficiency of the vitrification of human ES cells, a modified vitrification method has been devised (Reubinoff *et al.*, 2001). During vitrification, an instantaneous reduction of the temperature of human ES cells is necessary (more than ~1500℃ /min) to prevent the occurrence of ice crystallization, and to reduce cytotoxic effects through a high cryoprotectant concentration. A variety of vehicle types were analyzed in an attempt to enhance the survival rate and retainability of undifferentiated human ES cells. Among these vehicles, the OPS was the first vehicle type applied to the freezing of human ES cells, and remains the most popular method (Reubinoff *et al.*, 2001). The cooling rate and the types of cryoprotectants utilized have been recognized as important factors during vitrification using OPS (Richards *et al.*, 2004).

The EM-grid, when utilized as a vehicle, minimizes cell damage, as a consequence of its efficient thermal conductivity (Hong *et al.*, 1999). In the case of human ES cells, however, the EM-grid could carry only minimal numbers of human ES clumps (10 clumps), and evidences disadvantages with regard to storage, as it does not feature a container for the protection of human ES cells during cryopreservation. The micro-droplet vehicle method, which requires no apparatus, and was initially introduced for the cryopreservation of mammalian embryos in 2003, is a simpler freezing method than are the OPS and EM-grid methods (Papis *et al.*, 2000; Misumi *et al.*, 2003). The results of our study indicate that no significant difference exists between vehicles and micro-droplets with regard to the efficiency of recovery rates, survival rates, and the undifferentiation scores in human ES cells after thawing. These results indicate that the micro-droplet method may also be employed as another vitrification technique in the cryopreservation of human ES cells.

In conclusion, the results of this study reveal that vitrification is a more effective method than slow freezing in the cryopreservation of human ES cells. In addition, we compared the efficiency of the cryopreservation of human ES cells between micro-droplet, OPS, and EM-grid systems, using the scoring system for the degree of undifferentiation of human ES cells after cryopreservation. Although no statistically significant differences were detected among the vehicle types, the ES cells cryopreserved using OPS evidenced higher survivability as compared to other vehicle groups. Finally, we confirmed that human ES cells retained their pluripotency and normal karyotypes during prolonged culturing after thawing by vitrification. These data corroborate the possibility of the application of a variety of vehicles, including micro-droplet, OPS, and EM-grid techniques for the cryopreservation of human ES cells.

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