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Simplified Slow Freezing Program Established for Effective Banking of Embryonic Stem Cells

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ABSTRACT : This study was designed to simplify a cryopreservation program for embryonic stem cells (ESCs) by selection of cooling method and cryoprotectant. Commercially available mouse E14 embryonic stem cells (ESCs) were cryopreserved with various protocols, and morphology and viability of the frozen-thawed ESCs and their reactive oxygen species (ROS) production were subsequently monitored. Post-thaw colony-formation of ESCs was detected only after a slow freezing using dimethyl sulfoxide (DMSO) by stepwise placement of a freezing container into a -80°C deep freezer and subsequently into -196°C liquid nitrogen, while no proliferation was detected after vitrification. When the simplified protocol was employed, the replacement of DMSO with a mixture of DMSO and ethylene glycol (EG) further improved the post-thaw survival. ROS generation in ESCs frozen-thawed with the optimized protocol was not increased compared with non-frozen ESCs. The use of fresh mouse embryonic fibroblasts as feeder cells for post-thaw subculture did not further increase post-thaw cell viability. In conclusion, a simplified slow-freezing program without employing programmable freezer but using DMSO and EG was developed which maintains cell viability and colony-forming activity of ESCs during post-thaw subculture. (**Key Words :** Mouse, Embryonic Stem Cells, Vitrification, Slow Freezing, Cryoprotectant, Reactive Oxygen Species)

INTRODUCTION

An effective cryopreservation program for embryonic stem cells (ESCs) is essential for establishing ESC bank (Gearhart, 1998), as well as for developing a strategy for resisting ESCs against extremely low temperature. Both vitrification (Reubinoff et al., 2001) and slow freezing protocols (Freshney, 1994; Ware et al., 2005; Yang and Hua et al., 2005) have successfully been developed for cryopreserving ESCs, but further improvement is necessary for overcoming current limitation of ESC cryopreservation. In the case of vitrification program, post-thaw survival of ESCs after vitrification is greatly reduced by severe osmotic effect and cryoprotectant toxicity, which resulted from the exposure of ESCs to high concentration of vitrification solution (Ha et al., 2005). On the other hand, conventional slow freezing program consisting of equilibration, pre-cooling and seeding, and cooling program before plunging into liquid nitrogen is time- and labor-consuming, which is further required to be simplified for effective freezing and thawing. Even in the case of undertaking optimal slow-freezing protocol, the freezing protocol can not avoid to forming intracellular ice crystal, which triggers solution effect and mechanical damage (Ha et al., 2005).

To overcome such limitation, we have designed a simplified slow freezing program for cryopreserving cells and tissues. The suggested program only takes the merits of slow freezing method; it does not utilize programmable freezer and freezing cells are simply placed into a -80°C deep freezer and liquid nitrogen, consequently. Furthermore, it does not employ multistep equilibration protocols of cryoprotectants, seeding and cooling, and step-wise dilution procedure of cryoprotectant after thawing. Using this alternative program, we have achieved post-thaw survival rate as much as 50%. Especially in the case of ESC freezing that employed only small number of colony-forming cells are provided for the cryopreservation, however, the simplified program ought to further be optimized for increasing post-thaw survival (Miyamoto et al., 2006).

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Consequently, we conducted to optimize a method of ESC cryopreservation program. In this study, it was examined whether three factors, freezing protocol (vitrification or simplified slow freezing), cryoprotectant [dimethyl sulfoxide (DMSO), ethylene glycol (EG), or both] and using of additional feeder cells for post-thaw subculture, could improve post-thaw survival and maintenance of ESCs. The effectiveness of the optimized protocol was evaluated by monitoring ESC morphology and viability, and reactive oxygen species (ROS) production after thawing.

MATERIALS AND METHODS

Experimental design

In experiment 1, the efficiency of a simplified slow freezing program was compared with that of vitrification. Morphology of ESCs cryopreserved with either the simplified protocol using DMSO or vitrification was monitored after the first post-thaw subculture. In experiment 2, the availability of different cryprotectants used for the simplified protocol in experiment 1 was evaluated. The post-thaw survival of ESCs after the freezing with 10% DMSO (Robertson, 1987), EG of two levels (10% and 20%) (Ha et al., 2005), or a mixture of 10% DMSO and 10% EG (Mitsui et al., 2003) was monitored. To know the net effects of cryoprotectant more in details, the viability of ESCs was monitored after being exposed to each cryoprotectant without freezing. In experiment 3, generation of ROS by ESCs frozen-thawed with the optimized protocol was compared with that in fresh ESCs or the ESCs frozen slowly with DMSO. In the final experiment, the viability of ESCs after freezing-thawing with the optimized protocol was evaluated after being subcultured on the feeder layer of non-frozen, fresh mouse embryonic fibroblasts (MEFs).

Preparation of ESCs for cryopreservation

Commercially available E14 ESCs were purchased from ATCC (Manassas, VA) and were maintained with our standard culture protocols (Cho et al., 2006). Before experimental treatment, the ESCs were subcultured commonly five times on MEFs feeder in a modified Dulbecco's minimal essential medium (DMEM; Gibco-Invitrogen, Carlsbad, CA) supplemented with 15% (v/v) fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 1,000 U/ml of mouse leukemia inhibitory factor (LIF; Chemicon, Temecula, CA), 0.1 mM β-mercaptoethanol (Gibco-Invitrogen), 1% (v/v) nonessential amino acids, 2 mM L-glutamine and 1% (v/v) antibiotics solution (a lyophilized solution of penicillin and streptomycin). All of the medium substrates were purchased from Sigma-Aldrich Corp. (St Louis, MO), otherwise stated. The osmolarity of the medium was adjusted within a range of 270-275 mOsm.

Cryopreservation of ESCs

Five-hundreds thousand ESCs were seeded into feeder layers consisting of 1.0×10^6 MEFs for the 5th subculture and at the end of the culture, both ESCs and MEFs were treated with 0.25% (v/w) trypsin-EDTA solution for 5 minutes. After washing by centrifugation, both ESCs and MEFs were provided for the cryopreservation and their cell number was counted using a hemocytometer. In the case of undertaking vitrification, DMEM supplemented with 2 M DMSO (Edwards Lifesciences, Irvine, CA), 1 M acetamide, 3 M propylene glycol (Reubinoff et al., 2001; Fujioka et al., 2004) and fetal bovine serum (FBS; 15%, v/v) was used as a vitrified solution. One million cells including ESCs were suspended in 200 μl of the vitrified solution and they were subsequently placed into 1.2-ml cryovials. To minimize time to expose ESCs to vitrified solution, the vials were immediately plunged into liquid nitrogen at -196°C. For thawing, the vials stored in liquid nitrogen were placed in a 37°C incubator after the addition of 200 μl medium prewarmed to 37°C.

In the case of slow freezing, DMSO (10%, v/v), EG (10% or 20%, v/v) or both was used as a cryoprotectant. ESCs exposed to one of the cryoprotectants were subsequently suspended 1 ml cryoprotective solution (DMEM supplemented with one of the cryoprotectants). The suspended cells were subsequently loaded into a 1.2-ml cryovial. The vials were placed in a freezing container (Nalgene, Rochester, NY) and the container was placed in a deep freezer maintained at -80°C for 24 h, which kept a cooling rate of -0.5°C/min. The cryovials were then plunged directly into liquid nitrogen. For thawing, the cryovials were placed in a 37°C incubator and the pre-warmed medium was immediately added at the time of thawing.

Assessment of ESC viability

Post-thaw survival and subsequent proliferation of ESCs were evaluated by a colorimetric cell counting kit (CCK)-8 (Dojindo Molecular Technologies Inc., Gaithersburg, MD) assays. Three thousands cells suspended in 0.2 ml of DMEM supplemented with 15% (v/v) FBS were placed in one well of a 96-well plate dish, in which 2.0×10^4 non-frozen, MEFs were present as feeder cells or not. The viability of the cells after cultured for designated times was monitored by measuring absorbance at 490 nm using the Bio-Rad microplate reader Model-550 (Bio-Rad, Hercules, CA).

Measurement of ROS

Intracytoplasmic ROS concentration was measured by a

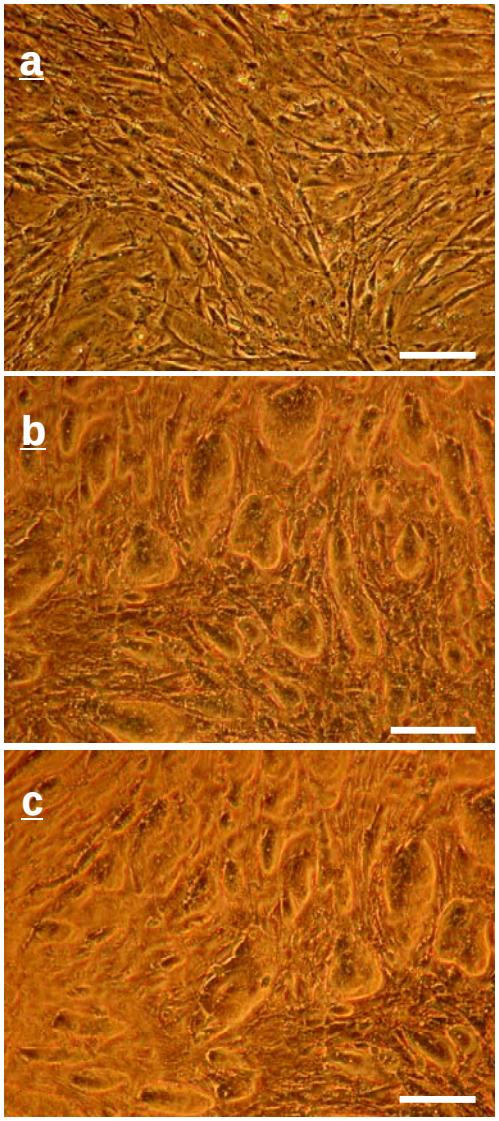

Figure 1. Colony formation of mouse embryonic stem cells (ESCs) after cryopreservation with different methods. Mouse E14 ESCs were cryopreserved with either (a) vitrification or (b) conventional slow freezing using 10% (v/v) dimethyl sulfoxide (DMSO). Morphological evaluation was made at the end of the first post-thaw subculture (on day 3 of thawing). As the control, ESCs without freezing and thawing (c) were simultaneously cultured with the cryopreserved ESCs. No colony formation was detected when ESCs were vitrified and thawed, while no significant difference in morphology was detected between the colonies derived from the non-frozen ESCs (control) and those derived from the ESCs frozen-thawed slowly. Scale bar = 100 μm.

method of Suzukawa et al. (Suzukawa et al., 2000) with slight modification. Briefly, the ESCs collected after different treatments were placed in a trypsin/EDTA solution and subsequently treated with 5 μg/ml 2',7' dichloro-dihydro-fluorescence diacetate (DCFHDA) at 37°C. Ten minutes after the treatment, 2.0×10^5 cells treated were placed in a 96-well plate dish and the DCF fluorescence intensity was measured by a multi-label microplate reader (Perkin Elmer, Victor 3) at 485 nm excitation wavelength and 530 nm emission wavelength.

Statistical analysis

All groups were replicated three times. A generalized linear model (PROC-GLM) in a Statistical Analysis System program was used to evaluate the effect of each group on cell proliferation and cell number counting. When analysis of variance (ANOVA) in the SAS package detected a significant main effect, groups were subsequently compared using the least square or DUNCAN methods. Significant differences among the treatments conducted were determined where the p value was less then 0.05.

RESULTS

Morphology of ESCs frozen with a simplified slow freezing protocol or vitrification

Apparent difference was detected in the morphology of ESCs vitrified or frozen slowly with DMSO (Figure 1). No colony-formation was detected in the ESCs vitrified and thawed, while colony-formation was observed in the ESCs frozen-thawed slowly. No significant difference in colony-forming activity and relevant morphology was detected between non-frozen ESCs (control) and the ESCs frozen-thawed slowly.

Effect of different cryoprotectants on the viability of ESCs frozen-thawed slowly

As shown in Table 1, a significant ($p<0.0001$) model effect of treatments (kinds of cryoprotectant) was detected in all parameters (days of observation). Between non-frozen ESCs and the ESCs frozen slowly, there was no difference in the viability immediately after thawing. Regardless of cryoprotectants, however, less viability was detected in frozen ESCs than in non-frozen ESCs on day 2 (0.836 vs. 0.560-0.763) and day 3 (0,938 vs. 0.544-0.731). Among the frozen-thawed ESCs, the 20% EG or the 10% DMSO plus 10% EG yielded better survival than other cryoprotectants on day 1 (0.657-0.736 vs. 0.555-0.574) and day 2 (0.652-0.763 vs. 0.560-0.571) of culture. On day 3 of culture, the highest viability was detected in the DMSO+EG (0.731 vs. 0.544-0.575). As shown in Figure 2, exposure of ESCs to DMSO ($p = 0.5130$) or DMSO+EG ($p = 0.5612$) without cryopreservation did not significantly reduce the viability on day 0 (0.558-0.571) or day 3 of culture (0.774-0.819).

Generation of ROS by ESCs cryopreserved with an optimized program

As shown in Figure 3, a significant model effect was detected ($p<0.0001$). Large amount of ROS was produced by the ESCs frozen-thawed with 10% DMSO compared

Table 1. Post-thaw viability of mouse embryonic stem cells (ESCs) and mouse embryonic fibroblasts (MEFs) after freezing with slow freezing protocols using different cryoprotectants

Cryoprotectants	Viability (Mean±SD) of ESCs and MEFs on			
	Immediately after thawing	Day 1	Day 2	Day 3
Control (no freezing)	0.747±0.051^{bc}	0.761±0.044^{bc}	0.836±0.020^{b}	0.938±0.033^{b}
DMSO (10%)	0.679±0.008^{b}	0.574±0.013^{d}	0.571±0.020^{c}	0.570±0.018^{c}
EG (10%)	0.654±0.013^{b}	0.555±0.003^{d}	0.560±0.010^{c}	0.544±0.010^{c}
EG (20%)	0.840±0.117^{c}	0.736±0.152^{bc}	0.763±0.146^{c}	0.575±0.085^{c}
DMSO+EG (10% each)	0.745±0.18^{bc}	0.657±0.188^{cd}	0.652±0.178^{c}	0.731±0.143^{d}

DMSO = Dimethyl sulfoxide; EG = Ethylene glycol.

E14 ESCs and mitonycin-C treated MEFs were concomitantly frozen in a cryovial and to monitor post-thaw viability, a colorimetric cell counting kit-8 was used.

Model effects of treatments in each parameter (day of observation) were less than 0.0001.

[a] Both frozen-thawed ESCs and MEFs were cultured in the absence of MEFs.

[b, c, d] Different superscripts within the same parameter were significantly different, $p<0.05$.

with non-frozen ESCs. Compared with the control, however, the production was not significantly increased after freezing-thawing with DMSO+EG, which was less than that of the ESCs frozen with 10% DMSO.

Effects of coculturing of frozen-thawed ESCs on fresh MEFs feeder

As shown in Figure 4, in the ESCs frozen-thawed slowly, subculturing with additional fresh MEFs feeder did not significantly promote post-thaw viability (0.526 to 0.530; p = 0.0834). However, subculture of fresh ESCs with additional MEFs increased the viability (0.541 to 0.581; p = 0.0218) in non-frozen ESCs.

DISCUSSION

The results of this study demonstrate that a simplified slow freezing program skipped protocols of cryoprotectant equilibration and dilution, seeding and cooling, and even without using of programmable freezer can be employed for long-term preservation of ESCs. Improved post-thaw cell survival with maintaining colony-forming activity and reduced production of ROS to that of non-frozen ESCs were detected after employing with the simplified protocol used a mixed cryoprotectant of DMSO and EG.

Not many reports on cryopreserving animal ESCs such as mouse (10-20% DMSO) (Robertson, 1987), chicken (7.5% DMSO) (Young et al., 1991), chum salmon (10% DMSO) (Kusuda et al., 2002) and rhesus monkey (10-20% DMSO) (Baran and Ware, 2007) have been reported. Low survival of human ESCs has been reported when they are frozen slowly with DMSO (Yang and Hua et al., 2005; Katkov and Kim et al., 2006). Several cryopreservation methods have been developed for human ESCs; vitrification (Reubinoff et al., 2001; Zhao et al., 2001; Suemori et al., 2006) or programmable slow freezing (Yang and Hua et

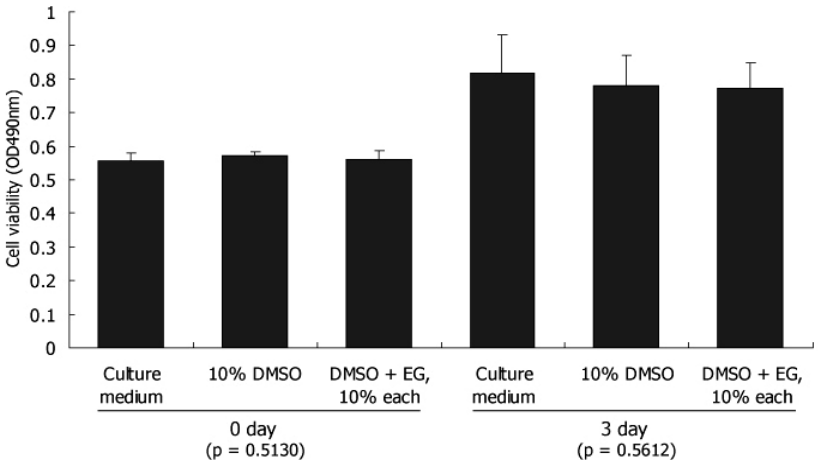

Figure 2. Viability of embryonic stem cells (ESCs) exposed to different cryoprotectants without freezing and thawing. ESCs of E14 cell line were co-cultured with MEFs monolayer for 3 days before exposure to cryoprotectant solutions of 10% (v/v) dimethyl sulfoxide (DMSO), 10% (v/v) ethylene glycol (EG), or both for 10 minutes. Cell viability was measured immediately after the exposure and 3 days after the treatments. Non-treated ESCs were provided as the control cells. Within the same day of observation, no significant difference was detected among the treatments ($p>0.513$).

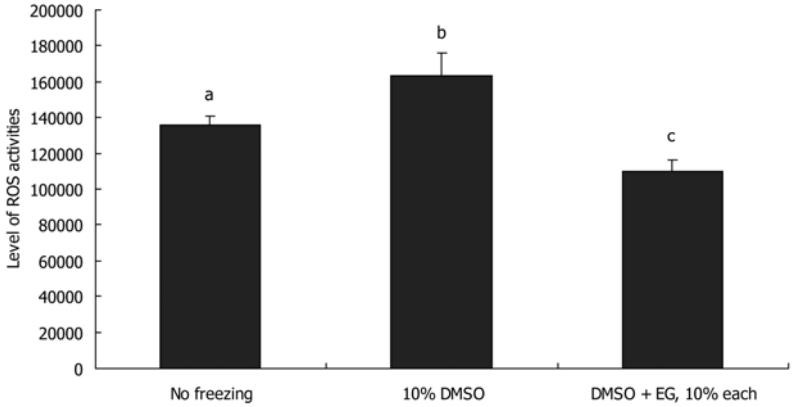

Figure 3. Production of reactive oxygen species (ROS) by mouse embryonic stem cells (ESCs) frozen-thawed slowly. E14 ESCs were slowly frozen with 10% (v/v) dimethyl sulfoxide (DMSO) or 10% DMSO and 10% ethylene glycol (EG), and intracytoplasmic ROS concentration was measured immediately after thawing. Non-frozen, fresh ESCs without cryopreservation were provided as the control treatment. Large amount of ROS was produced by the cells frozen-thawed with 10% DMSO compared with the levels produced by the non-frozen ESCs and by the ESCs frozen-thawed with DMSO+EG. Compared with the control, the ROS production of the cells frozen-thawed with DMSO+EG was not increased after freezing with 10% DMSO and 10% EG. Different letters in each parameter were significantly different ($p<0.05$).

al., 2005; Katkov and Kim et al., 2006). High concentration of cryoprotectants used for vitrification induces severe damages by cryoprotectant toxicity and osmotic damage to frozen-thawed cells (Reubinoff et al., 2001; Yang et al., 2003; Zhou et al., 2004). In this study, less efficiency on maintaining colony-forming activity of mouse ESCs was detected after vitrification than after slow freezing. Nevertheless, we are not able to deny the feasibility of vitrification program for effective cryopreservation of ESCs. There have been several reports to demonstrate the superiority of vitrification to other freezing programs for human ESCs, because it is able to avoid cell injury resulted from ice crystal formation (Reubinoff et al., 2001; Zhou et al., 2004; Suemori 2006). There are the numbers of factors affecting post-thaw survival of frozen-thawed (vitrified-thawed) cells, which do not consist of cryopreservation program itself (culture condition and cell viability before or after cryopreservation, etc.) and such factors may critically influence post-thaw viability. Otherwise, the ESCs maintained in our standard protocol may be vulnerable to the stress caused by vitrification used in this study.

Using of a simplified slow freezing program developed from our results can yield numerous benefits. First of all, our developed technique is handy and economical. In our

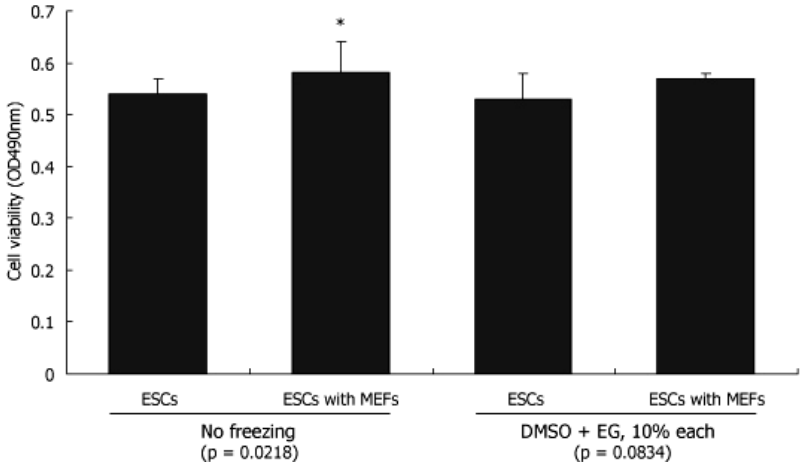

Figure 4. Post-thaw viability of mouse embryonic stem cells (ESCs) frozen slowly with dimethyl sulfoxide (DMSO) and ethylene glycol (EG). E14 ESCs either cocultured on feeder layer without freezing or frozen-thawed with DMSO+EG were subcultured with additional fresh MEFs treated with mitomycin C. On day 3 of culture, only ESCs were collected and monitored their viability using colorimetric cell counting kit-8. No significant model effect ($p = 0.0834$) was detected in the freezing group, while the significance ($p = 0.0218$) was detected in non-freezing group.

program, use of an isopropanol container enables to adjust cooling rate to -0.5°C/min without using of a programmable freezer by placing it in a deep freezer maintaining -80°C (Ha et al., 2005). There are no procedures on the equilibration and dilution of cryoprotectant in this program, but the selection of suitable cryoprotectants is essential. Cropreservation procedure ought to become different according to cell type and cellular characteristics. So, all components consisting of freezing and thawing procedures should specifically be determined in each case of crypreservation (Yi et al., 2002). In this study, the mixed cryprotectant of DMSO and EG yielded a better rate of post-thaw survival than others. Previous reports (Reubinoff et al., 2001; Ha et al., 2005) suggest that EG can improve survival of ESCs and the combined use of DMSO and EG stabilize cryopreservation outcome in human. EG is a lower molecular weight (62.1 g/M) than DMSO (78.1 g/M) and maintains rapid cell membrane permeability compared with other cryoprotectants. EG is known to be a comparatively safe compared with DMSO (Ball and Vo, 2001) and has low toxicity. Due to this nature, EG does not induce osmotic shock (Amorim et al., 2003). The results of this study showed that the combined use of EG with DMSO alleviates the toxic effect of DMSO to cryopreserved ESCs. However, results showing that use of EG alone did not effectively support the survival of ESCs after thawing implies that EG support cryoprotective action of DMSO (Reubinoff et al., 2001; Ha et al., 2005; Baran and Ware, 2007).

Both increase and decrease in ROS production beyond and below normal range, respectively, are detrimental to normal growth and proliferation of cells (Hancock et al., 2001). In this study, concomitant use of DMSO and EG as cryoprotectants maintains ROS production within normal range for survival. From different viewpoint, overproduction of ROS after being exposed to DMSO may be one of factors reduced cell survival after cryopreservation (Roca et al., 2004). Reactive oxygen species (ROS) are able to cause DNA damage and to induce lipid peroxidation. Those change membrane structure and fluidity, and inhibit enzyme activity linked to amino acid oxidation (Freeman and Crapo, 1982). It is not clear whether further decrease in ROS production by antioxidants can further enhance the post-thaw viability of ESCs.

Mitotically inactivated fibroblasts have been utilized for supporting growth of HeLa cells, mouse embryonal carcinoma cells and ESCs (Puck and Marcus, 1955), which secrete critical factors for cell maintenance (Ying et al., 2003; Ludwig et al., 2006). In addition to using MEFs for ESC cryopreservation, use of fresh MEFs for post-thaw culture of frozen-thawed ESCs did not significantly increase post-thaw survival of ESCs. Probably, mitotically inactivated MEFs concomitantly frozen-thawed with ESCs still maintain their function that is enough to support survival of ESCs after thawing. Reformation of MEFs monolayer was detected after the cryopreservation, while little delay to for monolayer compared with fresh MEFs was observed.

In conclusion, we suggest a simple cryopreservation method for effective freezing of ESCs. The suggested method contributes to stable long-term storage of ESCs with less effort. We now further evaluate the applicability of the established freezing program for ESCs of different origins and address whether further modification of the simplified protocol can improve post-thaw viability of ESCs.

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REFERENCES

Amorim, C. A., D. Rondina, A. P. Rodrigues, S. H. Costa, P. B. Goncalves, J. R. de Figueiredo and A. Giorgetti. 2003. Isolated ovine primordial follicles cryopreserved in different concentrations of ethylene glycol. Theriogenol. 60:735-742.

Ball, B. A. and A. Vo. 2001. Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential. J. Androl. 22:1061-1069.

Baran, S. W. and C. B. Ware. 2007. Cryopreservation of rhesus macaque embryonic stem cells. Stem Cells Dev. 16:339-344.

Cho, M., M. Jang, E. J. Lee, J. Y. Han and J. M. Lim. 2006. An alternative method of deriving embryonic stem cell-like clones by aggregation of diploid cells with tetraploid embryos. Fertil. Steril. 85:1103-1110.

Freeman, B. A. and J. D. Crapo. 1982. Biology of disease: free radicals and tissue injury. Lab. Invest. 47:412-426.

Freshney, R. 1994. Culture of animal cells, 3rd Ed. Wiley-Liss Press, New York, New York.

Fujioka, T., K. Yasuchika, Y. Nakamura, N. Nakatsuji and H. Suemori. 2004. A simple and efficient cryopreservation method for primate embryonic stem cells. Int. J. Dev. Biol. 48:1149-1154.

Gearhart, J. 1998. New potential for human embryonic stem cells. Sci. 282:1061-1062.

Ha, S. Y., B. C. Jee, C. S. Suh, H. S. Kim, S. K. Oh, S. H. Kim and S. Y. Moon. 2005. Cryopreservation of human embryonic stem cells without the use of a programmable freezer. Hum. Reprod. 20:1779-1785.

Hancock, J. T., R. Desikan and S. J. Neill. 2001. Role of reactive oxygen species in cell signalling pathways. Biochem. Soc. Trans. 29:345-350.

Katkov, II, M. S. Kim, R. Bajpai, Y. S. Altman, M. Mercola, J. F. Loring, A. V. Terskikh, E. Y. Snyder and F. Levine. 2006. Cryopreservation by slow cooling with DMSO diminished production of Oct-4 pluripotency marker in human embryonic stem cells. Cryobiol. 53:194-205.

Kusuda, S., T. Teranishi and N. Koide. 2002. Cryopreservation of chum salmon blastomeres by the straw method. Cryobiol. 45:60-67.

Ludwig, T. E., M. E. Levenstein, J. M. Jones, W. T. Berggren, E. R. Mitchen, J. L. Frane, L. J. Crandall, C. A. Daigh, K. R. Conard, M. S. Piekarczyk, R. A. Llanas and J. A. Thomson. 2006. Derivation of human embryonic stem cells in defined conditions. Nat. Biotechnol. 24:185-187.

Mitsui, K., Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, M. Maruyama, M. Maeda and S. Yamanaka. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113:631-642.

Miyamoto, Y., S. Suzuki, K. Nomura and S. Enosawa. 2006. Improvement of hepatocyte viability after cryopreservation by supplementation of long-chain oligosaccharide in the freezing medium in rats and humans. Cell Transplant 15:911-919.

Puck, T. T. and P. I. Marcus. 1955. A rapid method for viable cell titration and clone production with hela cells in tissue culture: The use of X-irradiated cells to supply conditioning factors. Proc. Natl. Acad. Sci. USA. 41:432-437.

Reubinoff, B. E., M. F. Pera, G. Vajta and A. O. Trounson. 2001. Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method. Hum. Reprod. 16:2187-2194.

Robertson, E. J. 1987. Embryo-derived stem cell lines. In: Teratocarcinomas and embryonic stem cells: A practical approach (Ed. E. J. Robertson). IRL Press, Oxford, UK. pp. 71-112.

Roca, J., M. A. Gil, M. Hernandez, I. Parrilla, J. M. Vazquez and E. A. Martinez. 2004. Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. J. Androl. 25:397-405.

Suemori, H., K. Yasuchika, K. Hasegawa, T. Fujioka, N. Tsuneyoshi and N. Nakatsuji. 2006. Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. Biochem. Biophys. Res. Commun. 345:926-932.

Suzukawa, K., K. Miura, J. Mitsushita, J. Resau, K. Hirose, R. Crystal and T. Kamata. 2000. Nerve growth factor-induced neuronal differentiation requires generation of Rac1-regulated reactive oxygen species. J. Biol. Chem. 275:13175-13178.

Ware, C. B., A. M. Nelson and C. A. Blau. 2005. Controlled-rate freezing of human ES cells. Biotechniques 38:879-883.

Yang, B. C., G. S. Im, W. K. Chang, Y. K. Lee, S. J. Oh, D. I. Jin, K. S. Im and C. K. Lee. 2003. Survival and *in vitro* development of immature bovine oocytes cryopreserved by vitrification. Asian-Aust. J. Anim. Sci. 16:23-28.

Yang, P. F., T. C. Hua, H. C. Tsung, Q. K. Cheng and Y. L. Cao. 2005. Effective cryopreservation of human embryonic stem cells by programmed freezing. In: Proceedings of the 2005 IEEE Engineering in Medicine and Biology 27th Annual Conference Shanghai, China. pp. 482-485.

Yi, Y. J., Y. A. Kwon, H. J. Ko and C. S. Park. 2002. Effects of diluent component, freezing rate, thawing time and thawing temperature on acrosome morphology and motility of frozen-thawed boar sperm. Asian-Aust. J. Anim. Sci. 15:1553-1558.

Ying, Q. L., J. Nichols, I. Chambers and A. Smith. 2003. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115:281-292.

Young, H., D. Morrison, J. Martin and P. Lucas. 1991. Cryopreservation of embryonic chick myogenic lineage-committed stem cells. Meth. Cell Sci. 13:275-283.

Zhao, J., H. N. Hao, R. L. Thomas and W. D. Lyman. 2001. An efficient method for the cryopreservation of fetal human liver hematopoeitic progenitor cells. Stem Cells 19:212-218.

Zhou, C. Q., Q. Y. Mai, T. Li and G. L. Zhuang. 2004. Cryopreservation of human embryonic stem cells by vitrification. Chin. Med. J. (Engl) 117:1050-1055.