Medium composition for effective slow freezing of embryonic cell lines derived from marine medaka (*Oryzias dancena*)

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Abstract This study was conducted to identify optimal medium composition for freezing *Oryzias dancena* embryonic cell lines. Different freezing media consisting of various concentration of dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), and trehalose were prepared and long-term cultured embryonic cell line was frozen in each freezing medium by conventional slow freezing program for 7 days. Through measurement of viability and growth of post-thaw cells frozen in each freezing medium, it was determined that optimal composition of three

components was 10 % DMSO, 20 % FBS, and 0.1 M trehalose. The post-thaw cells frozen in optimal freezing medium showed similar morphology and growth rate with non-frozen cells. Next, this condition was applied to two different sets of experiment; (1) freezing of the same cells during expanded period (57 days) and (2) freezing of short-term cultured cells from other batches for 7 days. The viability of post-thaw cells was significantly low and comparable in set 1 and 2, respectively, when compared with the result of long term-cultured cells frozen in optimal freezing medium for 7 days and similar morphology and growth rate with non-frozen counterparts were detected in the post-thaw cells from both sets. In conclusion, this study first reports the optimal medium composition for freezing *O. dancena* embryonic cells, which can contribute to fish species preservation as well as improvement of cell-based biotechnology by providing stable cell storage.

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Introduction

Cryopreservation of cells makes it possible to establish a cell banking system that enables the researchers to manage a number of different kinds of cells for research, conservation, and transportation (Dash et al.

2008; Higaki et al. 2013; Mauger et al. 2006). For the purpose of management and preservation of fish biodiversity, cryopreservation technology for embryos, eggs, and sperm from various fish species has been developed (Kopeika et al. 2005). However, there has been limitations in successful cryopreservation of fish embryos and eggs due to their large size, large portion of yolk, high water content, and thick chorion (Chao and Liao 2001; Kobayashi et al. 2007; Strussmann et al. 1999). To overcome this limitation, developmentally-competent cells such as embryonic cells, primordial germ cells, and stem cells has been suggested as an great alternative to diploid embryos for cryopreservation (Kobayashi et al. 2007; Strussmann et al. 1999). In addition, high developmental potential of the cells can contribute to improvement of cell-based biotechnology like transgenic research (Bail et al. 2010; Yan et al. 2013). For successful achievement of such purposes, optimization of species-specific freezing condition for fish developmentally-competent cells should be preferentially implemented to provide stable cell storage and supply. To date, many studies have tried to optimize freezing condition of developmentally-competent cells from various fish species including zebrafish *Danio rerio* blastomeres and primordial germ cells (Higaki et al. 2013; Kopeika et al. 2005), medaka *Oryzias latipes* blastomeres (Strussmann et al. 1999), Rainbow Trout *Oncorhynchus mykiss* primordial germ cells (Kobayashi et al. 2007; Okutsu et al. 2006), whiting *Sillago japonica* blastomeres (Strussmann et al. 1999), pejerrey *Odontesthes bonariensis* blastomeres (Strussmann et al. 1999) and rohu *Labeo rohita* embryonic stem cells (Dash et al. 2008). Marin medaka, *Oryzias dancena*, is a good experimental model fish, like *D. rerio* and *O. latipes* that are well-known experimental model fish, because of several properties including daily-spawning, rapid growth resulting in short generation time, and simple management at laboratory scale (Lee et al. 2013a). In addition to these advantages, salinity tolerance from fresh water to seawater has made them a good marine model fish. Our previous study reported the establishment of *O. dancena* embryonic cell lines (Lee et al. 2013a) which show embryonic stem cell-like activities (unpublished data), but efficient freezing condition of them has not established yet. In this study, in order to develop efficient freezing condition of *O. dancena* embryonic cell lines, we investigated optimal composition of

freezing medium under conventional slow freezing program by employing two *O. dancena* embryonic cell lines; already-established one in a previous study (long-term cultured) and newly-established one in this study (short-term cultured). Different freezing media supplemented with various concentrations of dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), and trehalose were used to develop optimal freezing condition of embryonic cell lines and the viability and grow rate of frozen-thaw cells were measured to verify effectiveness of freezing condition.

Materials and methods

Cell culture

Two *O. dancena* embryonic cell lines were used in this study. One was a cell line at the 200th subculture that was already established and described in our previous report (long-term cultured; Lee et al. 2013a) and the other was prepared in this study as described previously and cultured to the 49th subculture (short-term cultured). Culture medium was a Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 4.5 g/L D-glucose, 20 mM HEPES, 1 % (v/v) non-essential amino acids (Gibco), 15 % (v/v) FBS (Cellgro, Manassas, VA, USA), 1 % (v/v) fish serum, 50 µg/ml embryo extract, 1 % (v/v) penicillin-streptomycin mixture (Gibco), 10 ng/ml recombinant human basic fibroblast growth factor (bFGF; Gibco), 100 µM β-mercaptoethanol (Gibco), 2 nM sodium selenite (Sigma-Aldrich, St. Louis, MO, USA), and 1 mM sodium pyruvate (Gibco). The fish serum and embryo extract were prepared as described previously (Lee et al. 2013a). The cells were cultured on 0.1 % gelatin (Sigma-Aldrich)-coated tissue culture plate with culture medium in a 28 °C incubator with an air atmosphere and sub-cultured every 2 or 3 days when they reached 80–90 % confluency.

Freezing and thawing of embryonic cells

For freezing of embryonic cells, cultured cells were washed two times in Dulbecco's phosphate-buffered saline (DPBS; Gibco), trypsinized by 0.05 % trypsin-EDTA (Gibco), and harvested by centrifugation (400 g, 4 min). Cell pellets were suspended with freezing medium composed mainly of DMEM (Gibco)

supplemented with various concentrations of DMSO (0, 10, 20 and 40 %; Sigma-Aldrich), FBS (0, 20, 40 and 60 %; Cellgro), and/or trehalose (0, 0.1, 0.2 and 0.4 M; Wako, Osaka, Japan). From cell suspension, 1×10^6 cells were transferred into 1.2 ml cryogenic vials (Sigma-Aldrich), which were subsequently transferred into a freezing container (Thermo Scientific, Vernon Hills, IL, USA) that had a cooling rate of $-1^\circ\text{C}/\text{min}$. After 12 h in deep freezer at -75°C , cryogenic vials were stored within liquid nitrogen (-196°C) for 7 or 57 days. For thawing of frozen embryonic cells, the cryogenic vials were placed into a water bath at 37°C for 2 min. After ice crystals have disappeared, cell suspension was transferred into 15 ml tubes (SPL life Sciences, Pocheon, Korea) with 3 ml of culture medium and the thawed cells were harvested by centrifugation at 400g for 4 min.

Viability and growth activity of frozen-thawed cells

To evaluate cell viability, 1×10^5 post-thaw cells were seeded immediately after thawing in a well of 96-Well Microplate (Thermo Scientific) with culture medium and the viability of them was evaluated using the cell counting kit-8 (Dojindo, Kushu, Japan), according to the manufacturer's instructions. Non-frozen cells were used as a control. The viability was calculated as $\text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}} \times 100$. To measure growth activity of frozen-thawed cells, 1×10^5 post-thaw cells were seeded in a well of 0.1 % gelatin-coated 24-well culture plate (SPL) filled with culture medium and cultured for 48 h in 28°C incubator with air atmosphere. After that, cultured cells were harvested using 0.05 % trypsin-EDTA (Gibco) and cell number was counted with a hemocytometer (Marienfeld, Lauda-Königshofen, Germany). These experiments were repeated three times in a independent manner.

Measurement of growth rate

For measurement of growth rate, first, 1×10^6 post-thaw cells were stabilized by being cultured in general cell culture condition during 48 h. Next, 2×10^4 post-thaw or non-frozen cells per well were seeded in 0.1 % gelatin-coated 48-well MultiDish (Thermo Scientific) filled with culture medium and cultured for 9 days in a 28°C incubator with air atmosphere. The culture medium was changed every 3 days after first change at

day 1 of culture. From day 1 to 9 of culture, proliferating cells were counted daily with a hemocytometer (Marienfeld). This experiment was repeated three times in a independent manner.

Statistical analysis

The Statistical Analysis System (SAS) software was used to analyze the numerical data. When an analysis of variance (ANOVA) identified a significant primary effect, treatments were subsequently compared by the least-square or Duncan's method. $P < 0.05$ were regarded as indicative of significant differences.

Results

Optimization of medium for freezing *O. dancena* embryonic cells

To identify optimal freezing condition of *O. dancena* embryonic cell lines, different concentrations of DMSO, FBS and trehalose were employed for the preparation of freezing medium that is basically composed of DMEM containing cryoprotectants (DMSO or trehalose) and FBS. First, 0, 10, 20 and 40 % DMSO were supplemented to freezing medium containing 40 % FBS and long-term cultured embryonic cells were frozen in each freezing medium. After 7 days, viability of frozen-thawed cells in each freezing medium showed significant differences (Fig. 1a, $p < 0.0001$), in which the highest viability ($64.3 \pm 12.6\%$) was detected in the cells frozen with 10 % DMSO. As next series of experiment, freezing medium containing 10 % DMSO was supplemented with 0, 20, 40, and 60 % FBS and long-term cultured embryonic cells were frozen in each freezing medium for 7 days. No significant difference was detected in cell viability among frozen-thawed cell groups (Fig. 1b; 42.3 ± 7.7 to $60.1 \pm 8.7\%$ in viability, $p = 0.0796$). Finally, 0, 0.1, 0.2, and 0.4 M trehalose were added to the freezing medium which had fixed concentrations of 10 % DMSO and 20 % FBS and the same parameter was evaluated. The cells frozen with 0.1 M trehalose showed the highest cell viability comparing with the other groups ($73.5 \pm 10.2\%$ vs. 31.9 ± 12.3 to $57.9 \pm 11.9\%$, $p < 0.0001$), but rather high concentration (0.4 M) of trehalose significantly inhibited the viability of post-thaw cells.

Fig. 1 Determination of optimal medium composition for freezing *Oryzias dancena* long-term cultured embryonic cells. The cells were frozen in the freezing medium containing different concentration of dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), or trehalose for 7 days and post-thaw cell viability was measured by a cell counting kit-8. Non-frozen cells were employed as a control and viability was calculated as $\text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}} \times 100$. The values indicate mean $\pm$ SD. **A** Cell viability according to different DMSO concentrations in freezing medium containing 40 % FBS. Significant model effect ($p < 0.0001$) was detected and the highest survival was identified in the cells frozen with 10 % DMSO. **B** Cell viability according to different FBS concentrations in freezing medium containing 10 % DMSO. No significant difference was detected among treatment groups ($p = 0.0796$). **C** Cell viability according to different trehalose concentrations in freezing medium containing 10 % DMSO and 20 % FBS. Significant model effect ($p < 0.0001$) was detected and the highest survival was detected in the cells frozen with 0.1 M trehalose. **A–C** Different letters within a graph indicate significant differences

detected among the groups, it was identified that all frozen-thawed cells except for two groups retained growth potential.

The post-thaw cells that were frozen with freezing medium containing 10 % DMSO, 20 % FBS, and 0.1 M trehalose were further cultured and compared with non-frozen cells on cell morphology and growth rate. As shown in Fig 3a, similar cell morphology was identified between before and after freezing-thawing. Doubling times of post-thaw and non-frozen embryonic cells were 26.64 and 26.24 h, respectively, not showing the difference between two cell populations (Fig. 3b).

Validation of optimal freezing condition

To validate optimal freezing condition employing 10 % DMSO, 20 % FBS, and 0.1 M trehalose, two different experimental sets were designed; (1) long-term cultured embryonic cells were frozen during expanded period of 57 days and (2) short-term cultured embryonic cells from another batch were frozen for 7 days in the same freezing condition. As the results, 50.7 ± 6.8 and 57.7 ± 8.3 % viabilities of post-thaw cells were identified in set 1 and set 2, respectively (Fig. 4a). When compared with a previous result of long-term cultured cells frozen during 7 days, the cells frozen during expanded period showed significantly lower viability (73.5 ± 10.2 vs. 50.7 ± 6.8 %, $p < 0.05$) while short-term cultured

Growth of post-thaw embryonic cells

To confirm the results of cell viability after freezing-thawing and to identify whether post-thaw cells retain growth potential, cell numbers of frozen-thawed cells from all treatment groups that employed DMSO, FBS, and trehalose were counted after culture for 2 days in the general culture condition (Fig. 2). As expected, 0 and 40 % DMSO groups under 40 % FBS that had very low post-thaw cell viability did not grow at all and 0.1 M trehalose group under 10 % DMSO and 20 % FBS that had the highest post-thaw cell viability showed significant higher cell number than the others ($30.7 \pm 3.1 \times 10^4$ vs. 0 to $26.5 \pm 2.29 \times 10^4$ cells, $p < 0.0001$). Although different cell numbers were

Fig. 2 Growth of post-thaw *Oryzias dancena* long-term cultured embryonic cells. The 1×10^5 post-thaw cells from all treatment groups that employed dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), or trehalose were cultured for 2 days and cell number of each was counted. The values indicate mean $\pm$ SD. All post-thaw cells except for two groups (0 and

40 % DMSO groups under 40 % FBS) were able to grow in culture and the highest cell retrieval was achieved in the post-thaw cells that were frozen with the freezing medium containing 10 % DMSO, 20 % FBS, and 0.1 M trehalose. Model effect was <0.0001 and *a–h* different letters indicate significant differences

Fig. 3 Comparison of cell morphology and growth activity between non-frozen and post-thaw cells. The long-term cultured embryonic cells were frozen in freezing medium containing 10 % dimethyl sulfoxide, 20 % fetal bovine serum, and 0.1 M

trehalose for 7 days and their morphology (**A**) and growth rate (**B**) after thaw were compared with non-frozen cells. Two cell groups did not show any difference in both morphology and growth rate. Scale bar = 50 μ m

cells of a different batch showed no significant difference in post-thaw cell viability (73.5 ± 10.2 vs. 57.7 ± 8.3 %, $p > 0.05$). The cell morphologies of post-thaw cells were similar with those of non-frozen cells in both post-thaw cell populations from two experimental sets (Fig. 4b) and likewise, growth rates were not significantly different between before and after freezing-thawing in both cell population (Fig. 4c, d).

Discussion

In the present study, optimal medium composition in slow freezing program of long-term cultured embryonic cell line from *O. dancena* was determined, which was subsequently confirmed with the same cells frozen during expanded period and in another batch of short-term cultured embryonic cells. The optimal composition of freezing medium was DMEM supplemented

Fig. 4 Validation of optimal freezing condition employing 10 % dimethyl sulfoxide, 20 % fetal bovine serum, and 0.1 M trehalose. Two different sets of experiment were designed; (1) freezing of long term-cultured cells during expanded period (57 days) and (2) freezing of short term-cultured cells from a different batch for 7 days. **A** Comparison of post-thaw cell viabilities with those of long term-cultured cells frozen for 7 days. The post-thaw cell viabilities were significantly lower

and comparable in sets 1 and 2, respectively. **B–D** Morphology and growth rate of post-thaw cells. Similar morphologies were identified between before (B1 for set 1 and B3 for set 2) and after (B2 for set 1 and B4 for set 2) freezing-thawing from both sets. Scale bar = 50 μm. Similarly, post-thaw cells from both experimental sets showed comparable growth rate with non-frozen counterparts (C for set 1 and D for set 2)

with 10 % DMSO, 20 % FBS, and 0.1 M trehalose. In the slow freezing program of the cells, determining the composition of freezing medium is the most important task because the freezing medium carries out a key role to prevent cellular damage during the freezing process (Hunt 2011). Prevention against cryo-damage is actually accomplished by a cryoprotectant supplemented to freezing medium by suppressing the ice crystal

formation during freezing process (Fuku et al. 1992) and thus the choice of cryoprotectant and its concentration should be carefully determined. Additionally, the composition of it should be differentially applied to different species considering their different physiological properties. The most common one is DMSO, which is universally used for cryopreserving animal cells such as human embryonic stem cells (10 %; Lee and Lee

2011), mouse spermatogonial stem cells (10 %; Lee et al. 2013b), poultry primordial germ cells (10 %, Setioko et al. 2007), and fish embryonic stem cells (0.8 M; Dash et al. 2008). A similar result was derived from this study. Optimal concentration of DMSO in freezing medium for *O. dancena* embryonic cell lines was 10 %, in which sufficient number of cells for further use were able to be retrieved from a small number of post-thaw cells after 2 days of culture suggesting that slow freezing program employing DMSO can be well applied to *O. dancena* embryonic cell lines for effective cryopreservation. FBS is a major component of the freezing medium since it plays a key role in reducing oxidative stress (Freimark et al. 2011; Gutteridge and Quinlan 1993). In this study, addition of FBS to freezing medium under 10 % DMSO did not influence post-thaw cell viability. Nevertheless, we decided to use minimum FBS concentration (20 %) in the next series of experiment based on previous reports about usefulness of FBS in cell freezing (Marco-Jiménez et al. 2006). The effectiveness of FBS in the freezing medium was subsequently demonstrated in the experiment in which the growth of post-thaw *O. dancena* embryonic cells was measured. The absence of FBS under 10 % DMSO drawn significant lower cell number than the other FBS-containing groups under 10 % DMSO after post-thaw cells from each group were cultured for 2 days. These results suggest that the addition of FBS in freezing medium is undoubtedly required for stable freezing of embryonic cell lines from *O. dancena* species. It was reported that DMSO has cytotoxic effects by inducing the alteration of membrane fluidity and cytoskeleton (Brayton 1986; Hunt 2011; Katsuda et al. 1987; Miranda et al. 1978). To further improve the viability of post-thaw cells, we focused on trehalose, a disaccharide composed of two alpha-glucose units, that are an excellent stabilizer of membranes and proteins in dehydration stress (Morgan et al. 2006; Rudolph and Crowe 1985). As expected, trehalose supplementation significantly improved post-thaw cell viability up to 73.5 ± 10.2 %. This ameliorative effect of trehalose is also supported by several reports for cell cryopreservation (Aboagla and Terada 2003; Eroglu et al. 2001). Besides, it has been reported that there are many other components to be able to improve freezing efficacy of the cells. Treatment of the components that have antioxidant activity in freezing process can remove reactive oxygen species followed by stabilization of post-thaw cells (Uchendu et al.

2010). Such components include vitamin C and E (Uchendu et al. 2010; Cabrita et al. 2011), taurine (Martínez-Páramo et al. 2013), cysteine (Tuncer et al. 2010), and so on. In addition, it has been known that antifreeze proteins which can efficiently inhibit ice recrystallization could improve red blood cell survival during cryopreservation (Carpenter and Hansen 1992; Chao et al. 1996). Further experiments for those components will be helpful for improving post-thaw viability of the embryonic cell lines from *O. dancena*.

Successful freezing of early sub-cultured embryonic cells could be accomplished by the freezing medium optimized in this study. The embryonic cells at early subculture have the cellular properties more close to original cells, from which embryonic cells were derived, in the aspect of epigenetic state and chromosomal normality (Bork et al. 2010; Kanatsu-Shinohara et al. 2005). Thus, cryopreservation of such cells allows continuous use of relatively non-transformed cells for biotechnological applications. Moreover, the data suggests that optimal composition of freezing medium can be universally used in different batches of cells. Our results from the cells frozen during expanded period indicated that optimized composition of freezing medium developed can be effective even in long-term cryopreservation by showing cell survival rate of more than half (50.7 ± 6.8 %) in the frozen cells. However, a significantly lower cell viability was identified after freezing for 57 days than for 7 days. Theoretically, the cells can be cryopreserved during long periods without any cellular changes at -196 °C because phase changes of water do not occur at that temperature and thus the only damage source is direct ionizations by background radiation (Mazur 1988). Our data argue against which may be related to cellular instability caused by suboptimal culture conditions for *O. dancena* embryonic cells. Under such circumstance, cells may response sensitively to their microenvironment. In spite of using same protocol and careful cell management in all independent experiments, subtle difference in the initial condition before being frozen might provoke high variation at the end point. Suboptimal culture condition for *O. dancena* embryonic cells can be indirectly proven by relatively low post-thaw cell viability (73.5 ± 10.2 % in maximum) in comparison with those from higher vertebrates largely reaching more than 80 % (Ock and Rho 2011; Naaldijk et al. 2012; Lee et al. 2013c). Different sets of

experiments focusing on freezing duration and employing short-term cultured cells or another batch of *O. dancena* cell lines will provide more clear evidence. Nevertheless, the viable post-thaw cells after being frozen during expanded period still retained normal growth rate and the number of them was sufficient to be amplified in culture for further use.

In conclusion, we first demonstrate that *O. dancena* embryonic cells can be stably frozen for cryopreservation in optimal freezing medium consisting of 10 % DMSO, 20 % FBS, and 0.1 M trehalose. The results from this study not only can provide useful information for fish species preservation but also will contribute to development of cell-based biotechnology taking advantage of this kind of cells through effective cell storage and supply.

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