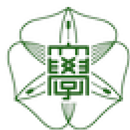

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FULL PAPER

Feasibility of cryopreservation of zebrafish (*Danio rerio*) primordial germ cells by whole embryo freezing

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Abstract

We investigated the feasibility of cryopreservation of zebrafish (*Danio rerio*) blastomeres and primordial germ cells (PGCs) by rapid freezing of dechorionated whole embryos at the blastula, gastrula and segmentation stages. Initially we examined the glass-forming properties and embryo toxicities of 5 cryoprotectants: methanol (MeOH), ethylene glycol (EG), dimethyl sulfoxide (DMSO), propylene glycol (PG), and 1,3-butylene glycol (1,3-BG). Embryos at the blastula and gastrula stages had high sensitivities to cryoprotectant toxicities and were fragile against mechanical damage. Thus the segmentation stage embryos, the PGCs of which were visualized by injecting green fluorescence protein-*nos1* 3'UTR mRNA, were frozen using solutions containing each cryoprotectant at 6 M (first trial) and 2 types of cryoprotectants at 3 M each (second trial). In the first trial, live PGCs were recovered from most of the embryos frozen with EG (about 2 cells/embryo); however, a few embryos had live PGCs when embryos were frozen with other cryoprotectants. In the second trial, a mixture of EG + PG better preserved the viability of PGCs in frozen embryos. Live PGCs were recovered from all embryos frozen with EG + PG (about 3 cells/embryo), and the survival rate of PGCs was estimated to be about 25% based on the number of live PGCs in fresh embryos (about 12 cells/embryo). The present study indicates that we can utilize rapid freezing of dechorionated whole embryos at the segmentation stage for the cryopreservation of PGCs.

Key words: blastomere, cryopreservation, embryo, primordial germ cell, zebrafish

Introduction

Cryopreservation of gametes and embryos is of great importance for preserving biodiversity, aquaculture and managing fish models used in biological research. Successful cryopreservation of fish spermatozoa has been achieved for many species[19,26]. Although successful cryopreservation of embryos has been reported for several fish species, including the zebrafish[8], common carp[41]

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and flounder[9,27], reproducible cryopreservation methods for fish oocytes and embryos have not been developed due to their large size, high sensitivity to chilling, and low membrane permeability[28,37].

Cryopreservation of blastomeres and primordial germ cells (PGCs) of embryos at the early developmental stage may provide a valid alternative to cryopreservation of gametes and embryos in fish[25,36] because functional sperm and oocytes can be obtained from germ-line chimeras that are produced by allogenic or xenogenic transplantation of blastomeres and PGCs (surrogate production). Surrogate production using cryopreserved blastomeres and PGCs is useful for preserving the genetic materials of endangered species and of domesticated fish strains carrying commercially valuable traits[25,36]. Germ-line chimeras induced by blastomere transplantation were reported in the rainbow trout[33], zebrafish[20] and medaka[34]. Survival of frozen-thawed blastomeres has been reported in zebrafish[10,21], rainbow trout[5,24], carp[6], chum salmon[17], goldfish[18], whiting, pejerrey and medaka[32] with slow freezing methods, and in zebrafish[7] with a rapid freezing method. However, there has been no report of production of germ-line chimera fish by transplanting frozen-thawed blastomeres.

Germ-line chimeras induced by PGC transplantation were also demonstrated in rainbow trout[33] and zebrafish[30]. In rainbow trout, germ-line chimeras have been produced by transplantation of PGCs originating from frozen-thawed genital ridges[15]. However, it seems difficult to obtain the genital ridge from relatively small embryos in non-salmonid fishes. For zebrafish, to our knowledge, cryopreservation of PGCs has not been studied, in spite of the importance of the zebrafish as a laboratory teleost fish model and vertebrate animal model[22].

The numbers of germ-line blastomeres and PGCs in zebrafish embryos are limited[23]; therefore, in the present study, we tried to develop a simple cryopreservation procedure that could avoid loss of PGCs during the freezing, thawing and handling of embryos. Considering the production of zebrafish germ-line chimeras by transplantation of frozen-thawed blastomeres and PGCs, we focused on the cryopreservation of blastomeres of blastula-stage (64- to 256-cell stage) embryos and PGCs in gastrula (50% epiboly)- and segmentation-stage (14- to 20-somite) embryos. This was because blastomeres in the embryos at later than the onset of gastrulation lose their pluripotency[11,12], and PGCs in the embryos at later than the 25-somite stage lose their ability to migrate to the genital ridge in recipient embryos[30]. Prior to embryo freezing experiments, we examined the embryo toxicities and glass-forming properties of cryoprotectants due to the lack of information about toxicities of cryoprotectants to zebrafish embryos at various developmental stages.

Materials and Methods

Preparation of fish embryos: Adult wild-type zebrafish (*Danio rerio*) were purchased from a local fish supplier (Scotto, Sapporo, Japan) and maintained at the Laboratory of Theriogenology, Graduate School of Veterinary Medicine, Hokkaido University. Up to 10 male and female zebrafish were kept separately in 12-l aquaria under a 14-hr light/10-hr dark cycle at 27 ± 1°C. The fish were fed three times a day with live brine shrimp nauplii (*Artemia franciscana*, INVE Aquaculture, Dendermonde, Belgium).

Embryos were obtained within 20 min after the initiation of the light period through natural mating. One female and two males were placed together in a mating cage with a mesh bottom that was prepared in a 12-l fish tank at 27 ± 1°C. The embryos were siphoned from the tanks into a collecting mesh and transferred to a plastic Petri dish (Falcon 1007, BD Biosciences, San Jose, CA, USA) filled with embryo medium[35] (EM: 13.7 mM NaCl, 0.54 mM KCl, 0.025 mM Na_2HPO_4, 0.044 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$) supplemented with 0.1% (w/v)

trypsin (from porcine pancreas, Sigma Aldrich, St. Louis, MO, USA), 0.1% (w/v) actinase E (protease from *Actinomyces* spp., Kaken Pharmaceutical, Tokyo, Japan) and 0.4% (w/v) urea (Nacalai Tesque, Kyoto, Japan). Within 30 min after the collection, embryos at the 1- to 8-cell stage collected in the dish were dechorionated by gentle pipetting with a hand-drawn glass Pasteur pipette (IK-PAS-9P, Iwaki Glass, Chiba, Japan) with a tip having a 900-μm inner diameter.

Some dechorionated embryos at the 1- to 8-cell stage were placed in agar-coated (1% agar in EM) plastic Petri dishes filled with EM and treated with green fluorescence protein (GFP)-*nos1* 3'UTR mRNA to visualize and identify their PGCs as described previously[29]. In brief, mRNA solution was injected into the yolk just under the blastodisc using a fabricated glass microinjection pipette with a tip having a 5-μm inner diameter. RNAs containing *nos1* 3'UTR exhibit rapid degradation in the soma, while being stabilized in the PGCs in which *nos1* is specifically expressed[16].

Dechorionated embryos with or without GFP treatment were placed in agar-coated plastic Petri dishes filled with EM and cultured at 28°C using an incubator (PCI-301, AS ONE, Osaka, Japan) until they developed to the 64- to 256-cell, 50% epiboly or 14- to 20-somite stage. The stage of embryonic development was identified according to the criteria described elsewhere[14]. GFP-treated embryos were checked for GFP fluorescence under an inverted microscope (TE300, Nikon, Tokyo, Japan) equipped with a GFP filter set. Embryos with GFP fluorescence and without any deformations were used. All embryo manipulations were carried out at room temperature (20 to 25°C).

All procedures described herein were conducted in accordance with the Hokkaido University guideline for the care and use of animals.

Glass-forming tendencies of cryoprotectants: Cryoprotectant solutions were prepared using cryoprotectants of analytical grade (Kanto Chemical, Tokyo, Japan) and EM. One microliter of cryoprotectant solution was loaded without embryos on a nylon mesh (100 μm pore size, NYTAL 13XX-100, SEFAR, Thal, Switzerland) that was cut into a strip shape (approximately 5 mm × 30 mm). The solution-loaded nylon mesh was immersed in liquid nitrogen. After storage in liquid nitrogen for at least 1 min, the frozen mesh was plunged into 10 ml of pure water (reverse osmosis-treated and deionized water; Milli-RX 12 Plus, Nihon Millipore, Tokyo, Japan) prepared in a 10-ml glass beaker at room temperature. Ice formation of cryoprotectant solutions during cooling and warming was checked by visual inspection for the presence of a milky appearance as described previously[31].

Embryo toxicities of cryoprotectants: Embryos were immersed in 10 ml of cryoprotectant solutions prepared in plastic Petri dishes. After 30-min exposure at room temperature, embryos were transferred to a plastic Petri dish filled with 10 ml of EM supplemented with 0.5 M sucrose and kept for 10 min at room temperature. They were washed 3 times with 10 ml of fresh EM in a plastic Petri dish, then placed in an agar-coated plastic Petri dish filled with EM and incubated for up to 24 hr in an incubator at 28°C. The survival of the embryos was assessed by their ability to develop into the heartbeat stage with spontaneous movement, a functional heartbeat, blood circulation and no signs of malformation.

Freezing and thawing of embryos: Embryos were frozen after serial exposure to equilibration solution (ES) and freezing solution (FS). The ES consisted of EM containing 0.35% (w/v) bovine serum albumin (BSA; fraction V, Sigma-Aldrich) and cryoprotectant(s) at 4 M. The FS consisted of EM containing 0.7% BSA and cryoprotectant(s) at 6 M. Both ES and FS (1 ml each) were prepared in individual wells of a 4-well dish (176740, Nalge Nunc International, Napersvill, IL, USA). Embryos were initially placed in ES for 30 min. They were then transferred into FS and kept for 10 or 20 min. After serial exposure to ES and FS,

5 embryos were loaded on a strip-shaped nylon mesh (about 5 mm × 30 mm: as described for the glass-forming test). The embryo-loaded nylon mesh was put on a filter paper to remove excessive FS before plunging it into liquid nitrogen. Ice formation during cooling of embryos was checked by visual inspection as described above (glass-forming tendency of cryoprotectant). The frozen nylon mesh was transferred into a cryogenic vial (AGC Techno Glass, Chiba, Japan) and stored in a liquid nitrogen tank for at least 1 day.

For thawing, the frozen nylon mesh was removed from the vial in liquid nitrogen and immersed in 10 ml of EM supplemented with 0.7% BSA and 0.5 M sucrose prepared in a 10-ml glass beaker. After 4-min immersion, embryos detached from the nylon mesh were transferred into 1 ml of dilution medium (EM supplemented 0.35% BSA and 0.25 M sucrose) and kept there for 5 min. They were then washed twice with 1 ml of fresh EM. Dilution medium and EM for washing were prepared in individual wells of a 4-well dish. All freezing and thawing procedures were done at room temperature.

Assessment of PGC viability: To determine the viability of PGCs, a single GFP-labeled fresh (control) or frozen-thawed embryo was transferred to a 10-µl droplet of Hank's premix solution[35] (138.28 mM NaCl, 5.42 mM KCl, 0.255 mM Na_2HPO_4, 0.445 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$) supplemented with 0.1% collagenase and 0.5% trypan blue in a plastic Petri dish (Falcon 1008, BD Biosciences). The embryo was partially digested in the droplet by pipetting using a hand-drawn glass Pasteur pipette with a tip having a 150-µm inner diameter. PGCs were identified by their bright GFP fluorescence and relatively large size compared to other embryonic cells as described previously[29]. The numbers of GFP-positive cells that were negative for trypan blue were counted under an inverted microscope equipped with a GFP filter set (Fig. 1A and B). All procedures were carried out at room temperature.

Experiment 1: glass-forming properties and embryo toxicities of cryoprotectants: We examined the glass-forming properties and toxicities of 5 cryoprotectants: methanol (MeOH), ethylene glycol (EG), dimethyl sulfoxide (DMSO), propylene glycol (PG) and 1,3-butylene glycol (1,3-BG). To determine the glass-forming properties of the cryoprotectants, 1 to 10 M cryoprotectant solutions were prepared at intervals of 1 M. Embryo toxicities of the 5 cryoprotectants were examined using embryos at the 64- to 256-cell, 50% epiboly and 14- to 20-somite stages.

Experiment 2: embryo freezing and PGC viability after thawing: In the first trial, GFP-labeled 14- to 20-somite-stage embryos were frozen using ES and FS containing each cryoprotectant at 4 and 6 M, respectively. In the second trial, GFP-labeled embryos at the 14- to 20-somite stage were frozen using ES and FS containing 4 and 6 M cryoprotectant mixtures, respectively (2 types of cryoprotectants at 2 and 3 M each, respectively). Ice formation of the FSs was also determined during cooling and warming of the solutions without embryos.

Statistical analysis: The rates of embryo survival after exposure to cryoprotectant solutions, ice formation in embryos during cooling, and embryos with live PGCs after thawing were analyzed by Fisher's exact test. Differences in the mean numbers of live PGCs were analyzed by Kruskal-

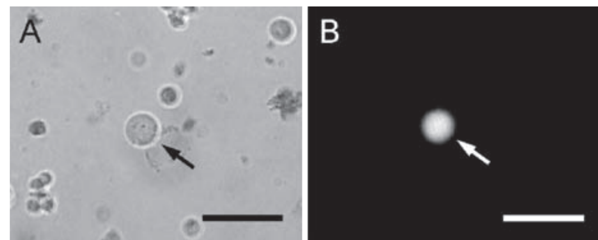

Fig. 1. A single PGC and other embryonic cells recovered from frozen-thawed embryos. Pictures were taken under light (A) and fluorescence (B) microscopy. Note that the trypan blue-negative PGC (arrow) is larger than other embryonic cells and exhibits intense green fluorescence. Bars = 50 µm.

Wallis analysis. The Mann-Whitney U test was used for comparisons between groups. For the statistical analyses, we used a computer program (SPSS for Windows, version 12.0, SPSS Inc., IL, USA).

Results

Experiment 1: glass-forming properties and embryo toxicities of cryoprotectants

As shown in Table 1, on a molar equivalency basis, the glass-forming properties of cryoprotectants were in the order MeOH < EG < DMSO < PG < 1,3-BG. As shown in Table 2, embryo toxicities of cryoprotectants depended on the embryonic developmental stage; the sensitivities of embryos to cryoprotectant toxicity were in the order 64- to 256-cell > 50% epiboly > 14- to 20-somite stage. When combining the results for embryos of the 3 developmental stages, the embryo toxicities of cryoprotectants were in the order EG > DMSO > MeOH > PG/1,3-BG.

Table 1. Glass-forming properties of cryoprotectants

Cryoprotectant	Lowest glass-forming concentration (M) during	
	Cooling	Warming
Methanol (MeOH)	10	> 10
Ethylene glycol (EG)	6	8
Dimethyl sulfoxide (DMSO)	5	6
Propylene glycol (PG)	4	6
1,3-Butylene glycol (1,3-BG)	3	6

Solutions containing 1 to 10 M concentrations of each cryoprotectant at intervals of 1 M were tested.

Experiment 2: embryo freezing and PGC viability after thawing

Recoveries of live PGCs from embryos frozen with various FSs are summarized in Table 3. Fresh (control) embryos had about 12 PGCs (Fig. 2A and B). In the first trial, live PGCs were recovered from most of the embryos frozen with EG (about 2 cells/embryo); however, a few embryos had live PGCs when embryos were frozen with other cryoprotectants. In the second trial, live PGCs were recovered from embryos in all experimental groups at various rates. FS containing EG + PG was superior to other FSs. All embryos frozen with EG + PG had live PGCs (about 3 cells/embryo: Fig. 2C and D) and the

Table 2. Embryo toxicities of cryoprotectants

Developmental stage	Concentration (M)	% of embryos survived after exposure to				
		MeOH	EG	DMSO	PG	1,3-BG
64- to 256-cell	2	10^{B}	0^{B}	0^{B}	60^{aA}	47^{aA}
	3	0	nd	nd	0^{b}	0^{b}
50% epiboly	2	100^{aA}	0^{B}	83^{aA}	100^{aA}	100^{aA}
	3	48^{bA}	nd	0^{bB}	60^{bA}	67^{bA}
	4	0^{c}	nd	nd	0^{c}	0^{c}
14- to 20-somite	2	95^{a}	90^{a}	92^{a}	100^{a}	100^{a}
	3	83^{aA}	0^{bB}	70^{aA}	85^{abA}	87^{abA}
	4	0^{bB}	nd	0^{bB}	77^{abA}	83^{abA}
	5	nd	nd	nd	62^{b}	74^{b}
	6	nd	nd	nd	12^{c}	39^{c}

Data were pooled from 3 to 5 replicates and 24 to 129 embryos. nd: no data.
^{A,B}Values with different superscripts within the same row differ significantly ($P < 0.05$).
^{a,b,c}Values with different superscripts within the same column of each developmental stage differ significantly ($P < 0.05$).

Table 3. Recoveries of live PGCs from embryos frozen with various freezing solutions (FS)

Freezing solution		Exposure to FS (min)	% of embryos with[b]		No. of live PGCs per embryo (n)	
cryoprotectant	ice formation[a]		ice formation	live PGC (s)		
Trial 1						
EG	−/+	10	0[b]	100[a]	2.1 ± 1.4[ab]	(10)
		20	0[b]	60[ab]	1.8 ± 0.8[bc]	(6)
DMSO	−/−	10	0[b]	30[bc]	1.3 ± 0.6[c]	(3)
		20	0[b]	0[c]	—	—
PG	−/−	10	100[a]	0[c]	—	—
		20	100[a]	30[bc]	1.0 ± 0.0[c]	(3)
1,3-BG	−/−	10	100[a]	0[c]	—	—
		20	100[a]	0[c]	—	—
Trial 2						
EG + PG	−/+	10	0[b]	100[a]	3.2 ± 1.8[a]	(10)
		20	0[b]	100[a]	2.8 ± 1.3[ab]	(10)
DMSO + PG	−/−	10	60[a]	70[ab]	1.4 ± 0.5[c]	(7)
		20	0[b]	100[a]	1.8 ± 0.8[bc]	(10)
EG + 1,3-BG	−/+	10	90[a]	20[bc]	1.0	(2)
		20	10[b]	70[ab]	2.0 ± 1.2[b]	(7)
DMSO + 1,3-BG	−/−	10	100[a]	20[bc]	1.5	(2)
		20	90[a]	70[ab]	1.3 ± 0.8[c]	(7)
Control		Fresh		100	11.5 ± 2.6	(10)

[a]Ice formation of FS without embryos during cooling/warming. [b]Ten embryos were frozen in each group.
[a,b,c]Values with different superscripts within the same column differ significantly ($P < 0.05$).

recovery rate of live PGCs was estimated to be around 25% based on the number of live PGCs in fresh embryos (about 12 cells/embryo).

Discussion

The lowest glass-forming concentrations of cryoprotectants during cooling observed in the present study (3 M 1,3-BG, 4 M PG, 5 M DMSO, 6 M EG and 10 M MeOH) were similar to the findings in previous studies in which cryoprotectant solutions were loaded in a 0.25- or 0.5-ml plastic straws (4 M PG, 5 M DMSO, 6 to 6.5 M EG and ≥10 M MeOH)[2,39].

The sensitivities of zebrafish embryos to cryoprotectant toxicity observed in the present study (64- to 256-cell > 50% epiboly > 14- to 20-somite stage) were similar to the findings in previous studies on zebrafish embryos: cleavage > epiboly > closure of blastopore[1]; 60% epiboly > 8-somite[3]. The toxicities of cryoprotectants to 64- to 256-cell (1,3-BG = PG < MeOH = DMSO = EG), 50% epiboly (1,3-BG = PG = MeOH < DMSO < EG) and 14- to 20-somite stage embryos (1,3-BG = PG < MeOH = DMSO < EG) observed in the present study generally agreed with the findings in previous studies on zebrafish embryos at the gastrula (MeOH = DMSO < EG)[40] and 6-somite stages (PG = MeOH = DMSO < EG)[39].

According to the results of glass-forming tests, in the first trial of Experiment 2, we used FS containing each cryoprotectant at 6 M; MeOH was excluded due to its low glass-forming prop-

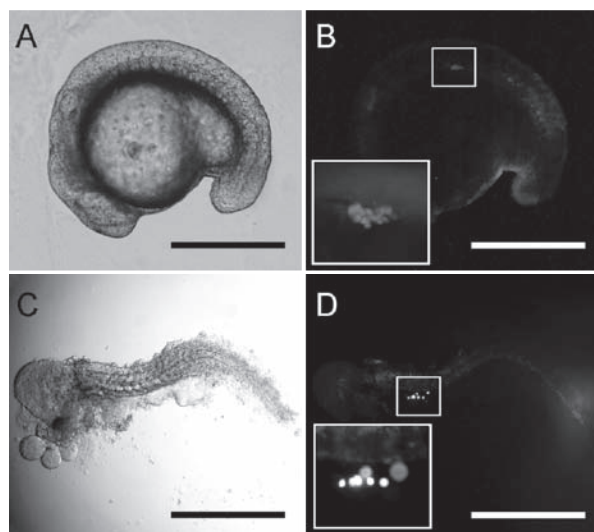

Fig. 2. Gross morphology of fresh and frozen-thawed embryos. A typical fresh embryo observed under light (A) and fluorescence (B) microscopy. Note the clear somite structure in the smooth surface blastoderm in A, and PGCs exhibit intense green fluorescence in B. A typical embryo frozen with EG + PG observed by light (C) and fluorescence (D) microscopy after about 10 min of thawing. Note the GFP-labeled PGCs in the blastoderm, which exhibit a clear somite structure despite the severe damage on the surface of the blastoderm. Bars = 500 μm.

erty. Embryos at the 14- to 20-somite stage were subjected to the PGC cryopreservation studies because embryos at the 64- to 256-cell and 50% epiboly stages were very sensitive to the cryoprotectant toxicities as shown in Experiment 1, and gave disappointing results in the preliminary trial. Embryos at the 64- to 256-cell stage were blotted into the filter paper during removal of excessive FS, and the blastoderm of embryos at the 50% epiboly stage totally dispersed into single blastomeres during thawing.

Although severe damage to the embryos was inevitable during freezing-thawing procedures under the present experimental conditions, some live PGCs were recovered from these damaged embryos. Survival of PGCs in the dead embryos indicated that a part of the blastoderm, in which PGCs are located, was successfully cryopreserved. In the first trial of Experiment 2, most embryos frozen with EG, and a few embryos frozen with other cryoprotectants, had live PGCs. Differences in the efficacies among cryoprotectants were not clear; however, the cryoprotective effects of cryoprotectants might depend on the rates of permeability, toxicity and glass-forming property. For the cryopreservation of cells, permeation of the cryoprotectant into the cells is essential, but is closely related to cytotoxicity[13]. Permeation rates of cryoprotectants in zebrafish embryos at the heartbeat stage were estimated to be in the order EG > DMSO > PG[37]. EG may permeate into embryos rapidly, and adequate permeation of EG into the deep peripheral endoderm, in which PGCs are located[4], may protect the PGCs from freezing injuries; however, rapid and excess permeation of EG may have cytotoxic effects in other compartments of embryos, leading to embryo death as shown in Experiment 1. DMSO, PG and 1,3-BG had higher glass-forming properties and low embryo toxicities; therefore, the low cryoprotective effects observed in FS containing these cryoprotectants might have been due to their low permeation rates.

FS containing 2 cryoprotectants, especially EG + PG and DMSO + PG, better preserved the viability of PGCs than FS containing the individual cryoprotectants that were used in the first trial. This might have been due to lowering of the toxicities of the cryoprotectants that had higher toxicities and permeabilities (*i.e.* EG and DMSO) and/or increasing the glass-forming property of FS by adding cryoprotectants that had lower toxicities and higher glass-forming abilities (*i.e.* PG and 1,3-BG).

The relationship between ice formation in embryos during cooling and the survival rate of PGCs was unclear. Recoveries of live PGCs from the embryos that showed ice formation during cooling indicated that induction of a complete glassy state (vitrification) of whole embryos was not required for the cryopreservation of PGCs. However, the reduction or prevention of ice formation in embryos is favorable for the cryopreservation of PGCs. Therefore, further experiments are needed to develop a reliable

freezing technique that can reduce or prevent the ice formation in embryos during cooling and thawing.

In conclusion, cryopreservation of blastomeres of blastula stage embryos and PGCs in the gastrula stage embryos was difficult under the present conditions; however, rapid freezing of dechorionated whole embryos at the segmentation stage can be utilized as a simple tool for the cryopreservation of PGCs. In the future, we need to clarify the developmental ability of PGCs recovered from frozen-thawed embryos by transferring them into surrogate embryos.

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