Successful cryopreservation of spermatogonia in critically endangered Manchurian trout (Brachymystax lenok)

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[1]

[1] **Abbreviations:** GSI, gonadosomatic index; NIBR, National Institute of Biological Resources; EG, ethylene glycol; PG, propylene glycol; Me_2SO, dimethyl sulfoxide; LN_2, liquid nitrogen; BSA, bovine serum albumin; GVC, Guava ViaCount; TB, trypan blue; dpt, days post-transplantation; SEM, standard error of the mean

Abstract

Because of the lack of cryopreservation techniques for fish eggs and embryos, cryopreservation of fish spermatogonia and subsequent generation of eggs and sperm would be an exit strategy for the long-term preservation of genetic resources. This study aimed to optimize cryoprotectants, cooling rates, and thawing temperatures for slow freezing of spermatogonia from endangered Manchurian trout (*Brachymystax lenok*). Whole testes were frozen with a cryomedium containing 1.3 M methanol, 0.2 M trehalose, and 10% egg yolk at a cooling rate of −1°C/min and then stored in liquid nitrogen for 2 days. After thawing at 30°C in a water bath, testicular cells from thawed testes were intraperitoneally transplanted into allogeneic triploid hatchlings. Transplanted spermatogonia migrated toward and were incorporated into recipient gonads, where they underwent gametogenesis. Transplantation efficiency did not significantly differ between frozen and fresh testes, demonstrating that Manchurian trout spermatogonia can be successfully cryopreserved in liquid nitrogen.

Manchurian trout, *Brachymystax lenok* Li 1996, is a landlocked salmonid fish that inhabits the upstream regions of East Asia and is listed as an endangered species in both Korea and China [8,9]. A subspecies of the Manchurian trout, *B. lenok tsinlingensis* has an extremely restricted distribution, for which the Nakdong River (Korea) defines the southernmost limit, where it has a small population size and suffers from habitat fragmentation because of the effects of climate change and habitat destruction [9]. This species and the regional population of Manchurian trout are seriously facing extinction; thus, measures for preserving genetic resources are urgently required.

Gamete or embryo cryopreservation could be an effective solution for the long-term preservation of genetic resources. However, fish eggs and embryos are too large to be successfully cryopreserved using current techniques [1,2,10]. Therefore, maternally inherited materials, including mitochondrial DNA, cannot be preserved using these techniques. We recently described a new method for deriving functional eggs and sperm from type A spermatogonia isolated from cryopreserved whole testes of rainbow trout [6]. This study aimed to establish a reliable and simple cryopreservation protocol for spermatogonia of the Manchurian trout.

Experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals by the National Institute of Biological Resources (NIBR; Incheon, Korea). Manchurian trout (*B. lenok*) were obtained from a local trout hatchery (Yangyang-gun, Korea) in May 2012 and maintained at NIBR to use as donor and recipient fish. Immature testes [testis weight, 0.019 ± 0.002 g; gonadosomatic index (GSI), 0.040% ± 0.003%] isolated from 10-month-old Manchurian trout donors (standard length, 12.3 ± 1.9 cm) were prepared for equilibrium slow freezing. Whole testes were transferred into 2 mL cryovials (Corning, Sigma-Aldrich) containing

1 mL cryomedium (pH 7.8) that comprised permeating cryoprotectants: methanol, ethylene glycol (EG), propylene glycol (PG), dimethyl sulfoxide (Me_2SO), or glycerol with 1.0, 1.3, or 1.6 M concentrations. We investigated four nonpermeating cryoprotectants: D-glucose, D-lactose monohydrate, D-(+)-trehalose dehydrate, or D-(+)-raffinose pentahydrate with 0.1, 0.2, or 0.3 M concentrations. We also tested 10% (v/v) fresh hen egg yolk vs. 1.5% (w/v) bovine serum albumin (BSA). The basal recipe of the cryomedium was reported previously [6]. The samples were equilibrated on ice for 60 min, then cooled at a rate of −0.5°C/min, −1°C/min, −10°C/min, or −20 °C/min to −80°C using a computer-controlled rate freezer (IceCube 14S; SY-LAB). After cooling, the samples were plunged into liquid nitrogen (LN_2) and were stored for 1 day, then thawed at 10, 20, 30, or 40°C in a water bath. Thawed testes were rehydrated in three changes of L-15 medium (Life Technologies, pH 7.8) containing 10% (v/v) FBS (Invitrogen). Extender was formulated as previously described [6]; all reagents for cryopreservation were purchased from Sigma-Aldrich unless otherwise stated.

To assess testicular cell viability, testes were trypsinized as previously described [7]. The cell suspension was filtered through a 42-μm nylon screen (N-No. 330T; Tokyo Screen Company, Japan) and re-suspended in Guava ViaCount (GVC) reagent (Guava Technologies, USA) to count viable cells using CytoSoft software (Guava Technologies, USA). Viable cells were also identified with the trypan blue (TB) exclusion test. On establishing that the total numbers of testicular cells did not significantly differ between both testes of a Manchurian trout ($39.8 \pm 3.0 \times 10^5$ vs. $41.0 \pm 2.2 \times 10^5$, $n = 4$, $P < 0.05$), the numbers of testicular cells in frozen and fresh testes were compared to determine cryopreserved testicular cell viability ($n = 4$–5). Viability was calculated as follows: viability (%) = [(GVC (+) + TB (−) cells in frozen testis)/(GVC (+) cells in fresh testis)] × 100.

To determine whether spermatogonia were recoverable from thawed testes, transplantation assays were performed as previously described [7]. Whole testes were equilibrated with cryomedium optimized in the preceding experiment and cooled at a rate of −1°C/min for 90 min using a slow-freezing container (CoolCell FTS30, USA) located in a −80°C freezer before being plunged into LN_2. After storage in LN_2 for 2 days, the cryovials were thawed at 30°C in a water bath. Testicular cells obtained from thawed testes were labeled with a fluorescent dye (PKH26 Cell Linker Kit, Sigma-Aldrich) to detect the donor cells in recipient gonads [5]; sterile triploid recipients were produced by heat shock of fertilized eggs at 28°C for 10 min subsequent to 15-min postfertilization and were then allowed to develop in environmental water at 10°C. Intraperitoneal transplantation was performed by microinjecting approximately 5 $\times 10^4$ PKH26-labeled cells (Fig. 2A and B) into hatchlings of triploid Manchurian trout (41–42 dpf). As control, cells harvested from fresh testes were also microinjected. At 25, 40, 151, and 558 days post-transplantation (dpt), the recipients were dissected; their gonads were examined with fluorescence microscopy (BX-53, Olympus). Because the transplantation efficiency [ratios at 21 dpt, 79.2% ± 4.0%: 77.8% ± 3.5%, $n = 33$, $P < 0.05$] did not significantly differ between the testes of a given Manchurian trout, the transplantation efficiencies of testicular cells from frozen and fresh testes were compared to determine the transplantability of cryopreserved testicular cells ($n = 15$–37). Ratios of recipients that possessed PKH26-labeled cells within their gonads at 25 and 40 dpt and the number of incorporated PKH26-labeled cells at 25 dpt were recorded. Ratios of recipients that possessed differentiating cells within their gonads were also examined at 151 and 558 dpt. The colonization, proliferation, and differentiation efficiencies of donor-derived spermatogonia in the recipient gonads were calculated by the formulae: colonization rate (%) = [(number of fish incorporating PKH26-labeled cells at 25 dpt)/(number of fish

observed)] × 100; proliferation rate (%) = [(number of fish proliferating PKH26-labeled cells at 40 dpt)/(number of fish observed)] × 100; differentiation rate (%)=[(number of fish having mature gonads at 558 dpt)/(number of fish observed)] × 100.

To determine the maturational stage of each gonad at 151 dpt, the middle portions of the gonadal fragments were fixed in Bouin's solution, embedded in paraffin, sectioned at 5-μm thickness, and stained with hematoxylin and eosin (H&E). Furthermore, to determine the ploidy level of recipients, blood cells were fixed in 70% (v/v) ethanol and incubated for 8 h in PBS (pH 7.8) that contained RNase A (10 μg/ml; Sigma) and propidium iodide (200 μg/ml; Sigma). DNA contents were analyzed using a Guava PCA-96 flow cytometry system (Millipore).

Data are presented as mean values ± standard error of the mean (SEM) derived from three independent experiments. Statistical significance was determined using the Student's *t*-test for comparisons between groups. For comparisons among more than two groups, statistical significance was determined using one-way ANOVA, followed by a Tukey test.

When whole testes were frozen at a cooling rate of −1°C/min and thawed at 10°C in a water bath, the viability of testicular cells frozen with cryomedium containing 1.3 M methanol was significantly higher than that frozen with cryomedium containing 1.3 M EG, 1.3 M PG, or 1.3 M glycerol (Fig. 1A). Of the testes frozen with cryomedium containing methanol or Me_2SO at 1.0, 1.3, or 1.6 M concentrations, the highest survival rate of testicular cells was obtained for those frozen with 1.3 M methanol (Fig. 1B). Nonpermeating cryoprotectants dissolved in cryomedium containing 1.3 M methanol were also assessed. The highest survival rate was observed for cells obtained from testes cryopreserved in cryomedium containing 0.1 M trehalose and 10% egg yolk (Fig. 1C). Of the testes frozen with cryomedium containing lactose or trehalose at 0.1, 0.2, or 0.3 M concentrations, cells obtained from those frozen with 0.2 M

trehalose demonstrated the highest survival rate (Fig. 1D). Next, the effects of cooling rates on cell viability were examined. Cooling rates of −0.5°C/min and −1°C/min produced significant increases in cell viability relative to other groups; the highest survival rate occurred with a cooling rate of −1°C/min (Fig. 1E). Cell viability of whole testes frozen at a cooling rate of −1°C/min with a cryomedium containing 1.3 M methanol, 0.2 M trehalose, and 10% egg yolk was assessed with thawing temperatures of 10, 20, 30, or 40°C. The highest survival of testicular cells (81.0% ± 1.3%) was obtained by thawing at 30°C in a water bath (Fig. 1F).

To determine whether spermatogonia possessing the ability to transdifferentiate into oocytes [6,7] were recovered from thawed testes, the transplantation efficiency was compared between frozen and fresh groups. Recipients were dissected at 25, 40, 151, and 558 dpt; PKH26-labeled donor cells were examined (Fig. 2B). Although red fluorescence was never observed in the gonads of 50 non-transplanted fish (Fig. 2C), frozen/thawed cells labeled with PKH26 were detected in the gonads of 88/104 recipients at 25 dpt (Fig. 2D); the cells rapidly proliferated in the gonads of 71/98 recipients at 40 dpt (Fig. 2F). Moreover, similar transplantation efficiencies were observed using freshly prepared PKH26-labeled cells (Fig. 2E and G). However, continued proliferation of PKH26-labeled cells resulted in a loss of fluorescence in gonads of all recipients at 151 dpt (0/97). Therefore, we performed histological analysis of each gonad at 151 dpt. In the non-transplantation group, the gonads of the triploid fish (6/6) contained only immature germ cells without advanced germ cells (Fig. 2H), whereas the ovaries of the female triploid recipients that received frozen spermatogonia (4/6) contained peri-nucleolus-stage oocytes and oogonia (Fig. 2I). Next, we examined gonads of recipients at 558 dpt, which were reared to the pre-spawning stage. As shown in Fig 2J, gonads in all 45 triploid fish that did not receive spermatogonia remained immature (gonad weight, 1.531 ± 0.240 g; GSI, 0.339 ± 0.062%); however, Fig. 2K

demonstrates that gonads in 38/61 triploid recipients that received frozen spermatogonia had maturing gonads (testis weight, 11.327 ± 2.312 g; GSI, 2.658% ± 0.570%; ovary weight, 9.708 ± 3.175 g; GSI, 2.355% ± 0.780%). Efficiencies of colonization (84.1% ± 7.4%), proliferation (72.3% ± 5.7%), and differentiation (60.7% ± 7.1%) and the numbers of incorporated spermatogonia (3.6 ± 2.0) did not significantly differ between the frozen and fresh groups (Table 1). All recipients were identified as triploids using flow cytometry (Fig. 2L), with the exception of five recipients in which triploidy induction failed; these five were not used in this study.

To save Manchurian trout from extinction, it is urgent to develop cryopreservation methods for long-term preservation of genetic resources. We successfully established a cryopreservation methodology for spermatogonia using the Manchurian trout, as evidenced by 81.0% viability of frozen testicular cells with the ability to derive vitellogenic oocytes and with transplantation efficiencies that did not significantly differ from the efficiencies for cells derived from fresh testes. To our knowledge, this is the first study to report cryopreservation methods for the Manchurian trout germ cells.

Previous studies distinguish live germ cells from somatic cells using *vasa*-GFP transgenic constructs [6,7] and GFP-*nos1* 3′UTR chimeric RNAs [3,4]; visualized germ cells were viability indicators in those studies. However, these techniques have not been developed for most endangered fish species, including the Manchurian trout. We determined viability by comparing results from frozen and fresh testes. Both sides of testes within a Manchurian trout were used because the total numbers of testicular cells and transplantation efficiencies did not significantly differ between the two testes. The method used can be directly applied to determine testicular cell viability within endangered fish species, although further investigation is required for different fish species.

Here, we optimized a protocol for the slow freezing of whole testes from Manchurian trout using cryoprotectants comprising 1.3 M methanol, 0.2 M trehalose, and 10% egg yolk. This protocol originated from a protocol used for testis cryopreservation in rainbow trout [6]. There may be large differences in how testes from different species respond to permeating cryoprotectants; namely, 1.3 M methanol and 1.3 M Me_2SO were the best permeating cryoprotectants for testicular cells of Manchurian trout and rainbow trout, respectively. Although it is well known that the optimal type of cryoprotectant is species specific [10], these results might be because the testes used for cryopreservation in this study (testis weight, 19 ± 2 mg) were larger than those in rainbow trout (testis weight, 14 ± 1 mg) [6]. Owing to the lower molecular weight of methanol, it penetrates Manchurian trout testes more rapidly than Me_2SO, and thus, the cryoprotectant molecules may reduce the intracellular ice formation leading to cell death. These results could have practical implications for the selection of optimal cryoprotectants for spermatogonial cryopreservation.

We recently initiated a project to cryopreserve whole testes of Manchurian trout trapped in the Nakdong River and believe this effort will significantly contribute to conservation and restoration of the endangered Manchurian trout.

Acknowledgments

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Figure Legends

Figure 1. Optimization of freezing conditions for testicular cells from the Manchurian trout. **(A)** Viability of testicular cells with cryomedium containing 1.3 M methanol, ethylene glycol (EG), propylene glycol (PG), dimethyl sulfoxide (Me_2SO), or glycerol. **(B)** Viability of testicular cells with cryomedium containing methanol or Me_2SO at 1.0, 1.3, or 1.6 M concentrations. **(C)** Viability of testicular cells with cryomedium containing 0.1 M glucose, 0.1 M lactose, 0.1 M trehalose, or 0.1 M raffinose with egg yolk or BSA. **(D)** Viability of testicular cells with cryomedium containing lactose or trehalose at 1.0, 1.3, or 1.6 M concentrations with egg yolk. **(E)** Viability of testicular cells at cooling rates of −0.5°C/min, −1°C/min, −10°C/min, or −20°C/min. **(F)** Viability of testicular cells after thawing at 10, 20, 30, or 40°C. Columns represent mean ± SEM (n = 4–5). Columns with different letters are significantly different with $P < 0.05$.

Figure 2. Transplantation of thawed testicular cells. (A,B) Thawed testicular cells labeled with red fluorescent dye (PKH26) in the bright-field **(A)** and fluorescent view **(B)**. **(C)** Gonad of a non-transplanted triploid fish as a control of D and E. **(D–G)** Frozen/thawed and freshly prepared PKH26-labeled donor cells were incorporated into the recipient gonads **(D,E)** and rapidly proliferated **(F,G)**. **(H,I)** H&E-stained histological section of gonads from a non-transplanted triploid fish **(H)** and ovaries from a female triploid recipient that received frozen spermatogonia **(I)**. **(J)** Immature ovary of a non-transplanted triploid fish as a control of K. **(K)** Triploid fish, which received frozen spermatogonia, had ovaries that possessed a large colony of differentiating oocytes. **(L)** DNA contents of a diploid Manchurian trout (upper panel) and triploid recipient (lower panel). Arrows indicate the gonads **(C–G,J,K)**. Scale bars, 20 μm **(A–H)**, 50 μm **(I)**, 2 mm **(J,K)**.

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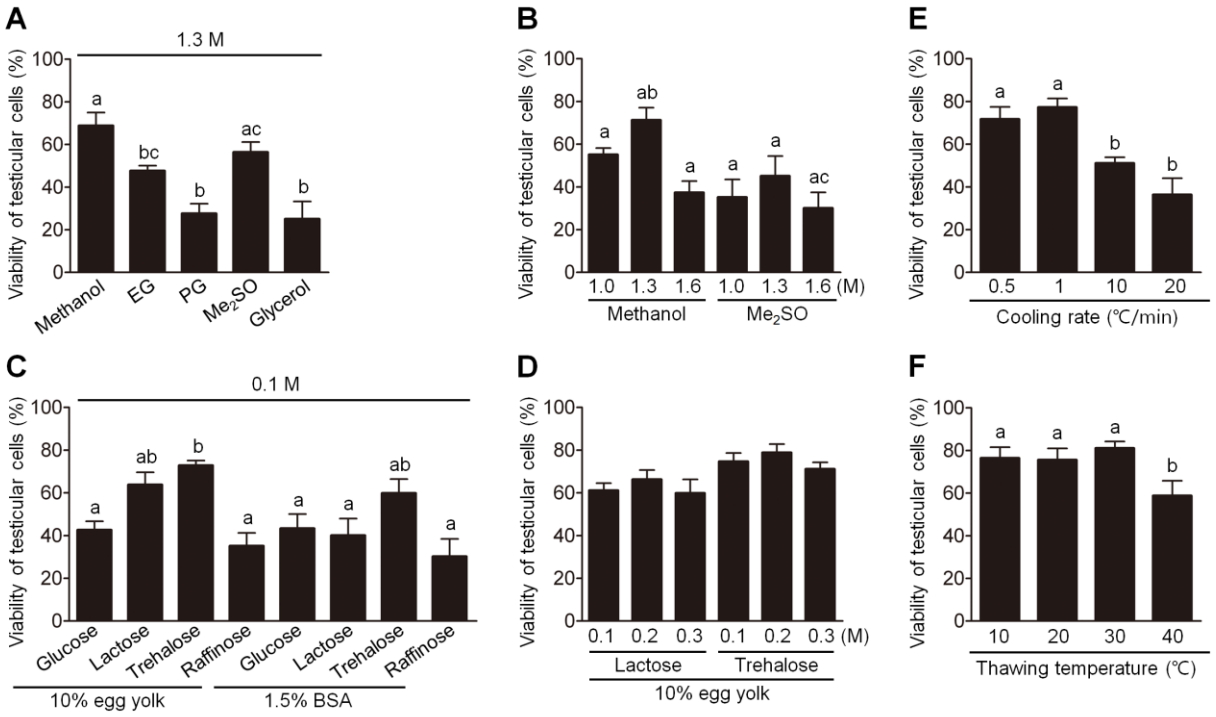
Fig. 1


A
1.3 M
Viability of testicular cells (%)
Methanol
EG
PG
Me2SO
Glycerol
B
Methanol
Me2SO
(M)
E
Cooling rate (℃/min)
C
0.1 M
Glucose
Lactose
Trehalose
Raffinose
10% egg yolk
1.5% BSA
D
Lactose
Trehalose
10% egg yolk
F
Thawing temperature (℃)

253 Fig. 2

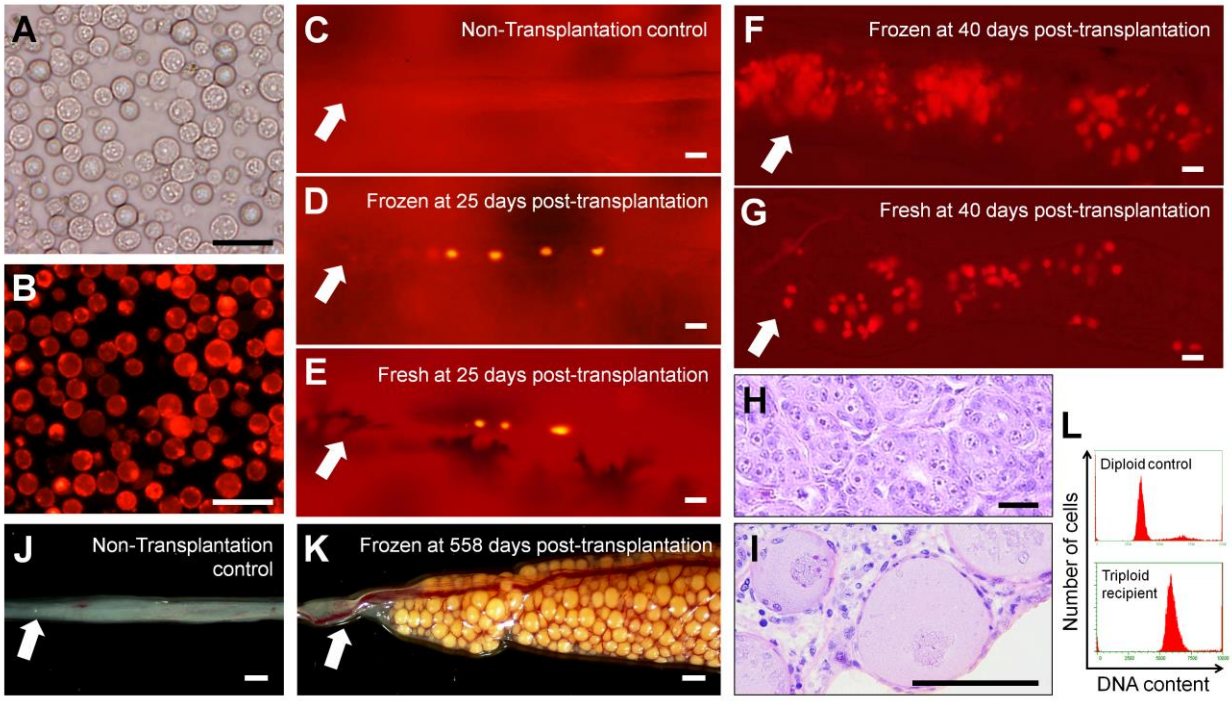


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Table 1. Colonization, proliferation, and differentiation of Manchurian trout spermatogonia in recipient gonads.

Group	No. of fish transplanted	No. of fish survived[d]	Colonization rate (%)	No. of colonized cells	Proliferation rate (%)	Differentiation rate (%)
Frozen[a]	105	104	84.1 ± 7.4[e]	3.6 ± 2.0[e]	72.3 ± 5.7[e]	60.7 ± 7.1[e]
Fresh[b]	100	98	89.0 ± 5.5[e]	4.5 ± 2.1[e]	75.0 ± 4.2[e]	53.2 ± 7.0[e]
Control[c]	50	50	0[f]	0[f]	0[f]	0[f]

[a] Triploid Manchurian trout recipients received spermatogonia cryopreserved for 2 days.

[b] Triploid Manchurian trout recipients received freshly prepared spermatogonia.

[c] Triploid Manchurian trout that did not receive spermatogonia.

[d] Number of viable recipients at 25 days post-transplantation.

[e,f] Values in a column with different superscripts are significantly different ($P < 0.05$).

Values are shown as mean ± SEM derived from three independent experiments.