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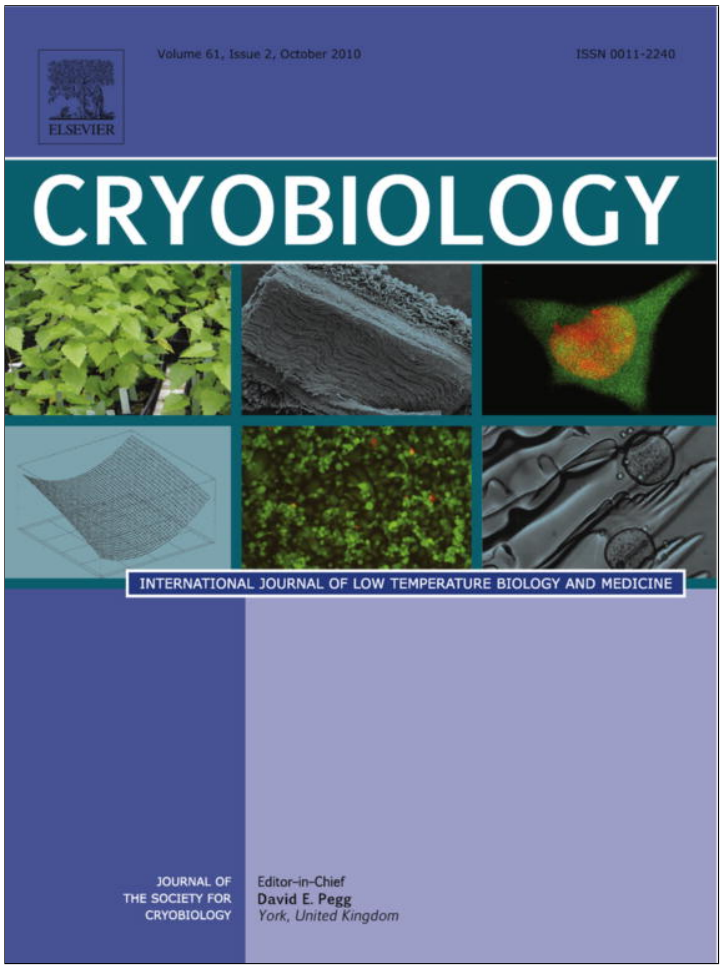

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Evaluation of cryoprotectant and cooling rate for sperm cryopreservation in the euryhaline “sh medaka Oryzias latipes☆

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ABSTRACT

Medaka Oryzias latipes is a well-recognized biomedical “sh model because of advantageous features such as small body size, transparency of embryos, and established techniques for gene knockout and modi“cation. The goal of this study was to evaluate two critical factors, cryoprotectant and cooling rate, for sperm cryopreservation in 0.25-ml French straws. The objectives were to: (1) evaluate the acute toxicity of methanol, 2-methoxyethanol (ME), dimethyl sulfoxide (Me $_2$SO), N,N-dimethylacetamide (DMA), N,N-dimethyl formamide (DMF), and glycerol with concentrations of 5%, 10%, and 15% for 60 min of incubation at 4 C; (2) evaluate cooling rates from 5 to 25 C/min for freezing and their interaction with cryoprotectants, and (3) test fertility of thawed sperm cryopreserved with selected cryoprotectants and associated cooling rates. Evaluation of cryoprotectant toxicity showed that methanol and ME (5% and 10%) did not change the sperm motility after 30 min; Me $_2$SO, DMA, and DMF (10% and 15%) and glycerol (5%, 10% and 15%) signi“cantly decreased the motility of sperm within 1 min after mixing. Based on these results, methanol and ME were selected as cryoprotectants (10%) to evaluate with different cooling rates (from 5 to 25 C/min) and were compared to Me $_2$SO and DMF (10%) (based on their use as cryoprotectants in previous publications). Post-thaw motility was affected by cryoprotectant, cooling rate, and their interaction (P6 0.000). The highest post-thaw motility (50 ± 10%) was observed at a cooling rate of 10 C/min with methanol as cryoprotectant. Comparable post-thaw motility (37 ± 12%) was obtained at a cooling rate of 15 C/min with ME as cryoprotectant. With DMF, post-thaw motility at all cooling rates was 6 10% which was signi“cantly lower than that of methanol and ME. With Me $_2$SO, post-thaw motilities were less than 1% at all cooling rates, and signi“cantly lower compared to the other three cryoprotectants (P6 0.000). When sperm from individual males were cryopreserved with 10% methanol at a cooling rate of 10 C/min and 10% ME with a rate of 15 C/min, no difference was found in post-thaw motility. Fertility testing of thawed sperm cryopreserved with 10% methanol at a rate of 10 C/min showed average hatching of 70 ± 30% which was comparable to that of fresh sperm (86 ± 15%). Overall, this study established a baseline for high-throughput sperm cryopreservation of medaka provides an outline for protocol standardization and use of automated processing equipment in the future.

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Introduction

The application of small-bodied “sh species including medaka Oryzias latipes as animal models in biomedical and environmental research has increased dramatically over the past two decades. Medaka has an extensive list of attributes including ease of laboratory culture, short generation time (4…6 weeks), proli“c reproduction, transparent embryos, wide tolerance of temperature and salinities, low basal rates of tumor formation, and responsiveness to a broad range of toxicant exposures that have contributed to the widespread use of this species [8,11,23,24]. Whereas various aspects of medaka biology, including physiology, genetics, and embryology have been studied for more than a century [25], recent advances in genomics and genetics research have intensi“ed interest in the use of medaka as a comparative animal model. The recent completion of the draft medaka genome sequence and subsequent releases of additional genome coverage [13] have contributed to the placement of medaka among the leading model organisms for genomics and developmental genetics research.

Advances in genetic modi“cation procedures, from the “rst transgenic medaka [20] to the most recent disruption (or knock-out) of

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speci“c gene function [21] have further increased the attractiveness of this species. In particular, procedures such as tilling (Targeted Induced Lesions In Genomes), in which random mutations are chemically introduced in the male genome followed by breeding with wild-type females to establish a library of genomic DNA and sperm from the F $_1$ generation male offspring hold significant promise in the use of medaka as an animal model [21] . These gene-targeted mutants are retrieved using in vitro fertilization with cryopreserved sperm, and the lineages are established by subsequent out crossing. The generation of gene knockout models by target-selected mutagenesis represents a signi“cant advance to develop new lineages of mutant medaka to explore mechanisms of gene function and related disease processes. The continued expansion of genetic resources including specialized lineages such as targeted gene knockouts and transgenics will contribute thousands of new lineages over the next decades. Coupled with the needs to preserve diminishing native populations of medaka, the demands for increased use of this species highlight the need to improve standardization, ef“ciency, and high-throughput capabilities for cryopreservation of medaka stocks.

To date, sperm cryopreservation of medaka has been reported in two publications [1,15] , and a recently published book chapter [14] based on the combination of these two publications. The “rst publication reported fetal bovine serum as an extender and 10% dimethyl sulfoxide (Me $_2$SO) and dimethylformamide (DMF) as cryoprotectants [1] ; higher fertilization was reported with DMF (96…100%) than that with Me $_2$SO (60%). The second publication reported 0.6 M sucrose as extender, 10% Me $_2$SO as cryoprotectant, and fertilization of 85% [15] . Whereas these protocols are currently used [14] , there remains a need to evaluate critical factors such as cryoprotectant, cooling rate, and male characteristics to improve procedures with an emphasis on increased ef“ciency, standardization, and potential for high-throughput applications. Also, the packaging used in these reports was plastic cryovials or glass capillary tubes. These containers lack capability for automated processing, which is desirable for establishment of large sperm repositories, and present inef“ciencies in labeling and storage. In addition, the cooling of samples was not quanti-“ed due to the nature of the methods used (i.e. suspension over dry ice, liquid nitrogen or liquid nitrogen vapor), and thus are dif“cult to repeat. Therefore, a standardized protocol that is readily reproducible among different laboratories is required for this species with assessment of the critical factors in cryopreservation using methods suitable for adoption in high-throughput approaches.

Generally, cryopreservation involves a series of steps including sample collection, sperm extension (dilution), cryoprotectant selection, cooling, cryogenic storage, thawing, and viability assessment [16] . Development of protocols for cryopreservation requires suitable choices at each step and consideration of the interactions among the factors. Inef“ciencies at each step can lead to considerable (cumulative) loss of viability in thawed sperm [16] . This study was intended to develop a protocol for medaka sperm cryopreservation based on evaluation of cryoprotectant and cooling rate. The objectives were to: (1) evaluate the acute toxicity of methanol, 2-methoxyethanol (ME), Me $_2$SO, N,N-dimethylacetamide (DMA), DMF, and glycerol with concentrations of 5%, 10%, and 15% for 60 min of incubation at 4 C; (2) evaluate cooling rates from 5 to 25 C/min for freezing and their interaction with cryoprotectants, and (3) test fertility of thawed sperm cryopreserved with selected cryoprotectants and associated cooling rates. In this study, commercially available 0.25-ml French straws were used for packaging of samples, enabling permanent labeling and reliable sealing; Hanks• balanced salt solution (HBSS, without protein addition) was used as extender, and a programmable freezer was used for precise control of cooling rates.

Materials and methods

Fish care

Medaka (obtained from Carolina Biological, derived originally from a strain native to Southern Japan), 6…8 months old, were obtained from the Aquatic Biotechnology and Environmental Laboratory of the University of Georgia (Athens, GA) by overnight shipping to Baton Rouge, LA. At the University of Georgia, the “sh were cultured in a freshwater (30 mOsmol/kg) system with a photoperiod of 16 h light: 8 h dark at 26 C with routine disease screening, and were fed 2…3 times daily with live Artemia larvae hatched from cysts (Brine Shrimp Direct, Ogden, UT) and commercial ”akes (Zeigler Aquatox Food or Otohime B1). Upon arrival, the “sh were maintained in 5-l tanks with 2…3 “sh/l in a freshwater aquarium system (Aquatic Habitats’, Aquatic Eco-systems, Inc., Apopka, FL), and were fed twice daily with commercial ”akes (Aquatic Eco-system, Apopka, FL) and live hatched Artemia larvae (INVE group; Grantsville, UT). The photoperiod was maintained at 14 h light: 10 h dark at 26 C. The water quality for the systems at both locations was: dissolved oxygen >6.5 mg/l; total ammonia-N <3.0 mg/l, nitrite-N <0.1 mg/l, and hardness (as Ca $_2$CO$_3$) >100 mg/l. Guidelines from the Institutional Animal Care and Use Committees of Louisiana State University Agricultural Center and University of Georgia were followed for animal care in this study.

Sperm collection and processing

Male “shes were anesthetized with tricaine methanesulfonate (MS-222, 0.05%, Western Chemical Inc., Ferndale, WA) or ice slurry for 1 min (to avoid MS-222 effects on sperm quality), and blotted with a paper towel to dry the body. Before dissection, standard length (from snout tip to the base of the caudal “n) and body weight were measured, and “sh were killed by severing of the spinal cord. The testes were removed from surrounding tissues while viewing with a dissecting microscope (10 magni“cation) and were transferred to tared 1.5-ml centrifuge tubes for weighing. Hanks• balanced salt solution (HBSS) with an osmolality of 350 mOsmol/kg (HBSS350) was used as extender in this study. The HBSS350 was prepared by adjusting the water volume using the recipe for HBSS300 (0.137 M NaCl, 5.4 mM KCl, 1.3 mM CaCl $_2$, 1.0 mM MgSO $_4$, 0.25 mM Na $_2$HPO$_4$, 0.44 mM KH $_2$PO$_4$, 4.2 mM NaHCO$_3$, and 5.5 mM glucose, pH 7.2), and the “nal osmolality was measured by a vapor pressure osmometer (model 5520, Wescor Inc., Logan, Utah). Sperm suspensions were collected by crushing testes in HBSS350 with a volume (l l) of approximately 40 times the testis weight (mg) yielding approximately 4…8 10^7 cells/ml (estimated from our preliminary data from 10 males for sperm concentration determination) except the samples in Experiment III in which the sperm concentrations were determined by use of computer-assisted sperm analysis (CASA) (Hamilton Thorne, Inc., Beverly, MA; CEROS model) while estimating the sperm motility (described below).

Motility estimation

To estimate motility in Experiments I and II, 2 l l of sperm suspension were placed on a glass slide, and 18 l l of HBSS with an osmolality of 100 mOsmol/kg were added to yield a “nal osmolality of 125 mOsmol/kg which activated medaka sperm motility [26] . The motility was observed at 200 magni“cation using a dark-“eld microscope (Optiphot 2, Nikon Inc., Garden City, NY, USA). The motility was expressed as the percentage of sperm that moved actively in a forward direction; sperm that vibrated in place were not counted as motile. For each sample, sperm motility was estimated

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at least two times with three “elds observed each time. For the sperm samples used for in vitro fertilization in this study (Experiment III), the motility was also evaluated by use of CASA with 20-l m 2-cell slides (Hamilton Thorne, Inc.). The parameter settings of CASA were: minimum contrast, 30; minimum cell size, 4; number of frames for recording, 60; average path (VAP) cut off, 15 l/s; straight line (VSL) cut off, 10 l/s; intensity gate, 0.10…1.68; cell gate, 0.72…2.25; elongation gate, 50…100, and slow cell, static.

Freezing and thawing procedures

Prior to cooling, the sperm samples were mixed with an equal volume of double-strength (of target concentration) cryoprotectant in HBSS350, and held on ice for 15 min of equilibration (de-“ned as the time from addition of cryoprotectant to sperm suspension to the time when samples were at 0 C during the cooling process). Aliquots (100 l) of sperm suspension were loaded into each 250-l French straw (IMV International, Minneapolis, MN, USA) by pipetting. The samples were cooled from 5 to 80 C at the speci“ed cooling rates (based on sample probe temperature) in a programmable freezer (Kryo 10 series II; Planer Products, Sunbury-on-Thames, UK), and transferred to liquid nitrogen for storage. Samples were evaluated after a minimum of 7 d storage in liquid nitrogen. Motility was estimated at Aquaculture Research Station of the Louisiana State University Agricultural Center. Fertility testing by in vitro fertilization was performed at the University of Georgia following transport of samples (coded for blind analysis) in a 10-l shipping dewar (LD-10, Taylor-Wharton, Theodore, AL). Each cryopreserved sperm sample was thawed individually in a water bath at 40 C for 5 s, wiped dry with a paper towel, and was released into a sterile 1.5-ml centrifuge tube by cutting of the end (with the cotton plug).

In vitro fertilization

In vitro fertilization was performed at the University of Georgia. Females were separated from males on the preceding day. Within the “rst 4 h following onset of light, females were killed with an overdose of 0.1% MS-222, dissected, and the ovary removed and placed in a dish with balanced salt solution for medaka (BSS: NaCl, 111.23 mM; KCl, 5.37 mM; $CaCl_2$, 1.80 mM; $MgSO_4\ 7H_2O$, 0.81 mM; $NaHCO_3$, 0.10 mM, pH 7.4) with an osmolality of 220 mOsmol/kg [12] measured by osmometer. While viewing with a dissecting microscope, mature eggs were identi“ed and removed from the ovarian tissues by use of “ne forceps, and single eggs were separated by cutting their attachment “laments near the surface of the chorion of each egg by use of small scissors. The eggs were held in BSS prior to fertilization for as long as 75 min. To perform in vitro fertilization, individual cryopreserved sperm samples were thawed and added to the eggs within 10 s. BSS (900 l) was added within 1 min to the egg-sperm mixture (990 l total solution volume). The fertilized eggs were incubated for 5 min at 26 C to allow the chorion to become rigid enough for handling. The fertilized eggs were transferred to egg-rearing solution (NaCl, 102.67 mM; KCl, 0.40 mM; $CaCl_2$, 0.27 mM; $MgSO_4$, 0.65 mM) in a Petri dish for incubation at 26 C for approximately 10…14 d until hatching.

Pooled eggs from 2 to 4 females were divided into two groups to provide matched samples for direct comparison of egg quality for each cryopreserved sample. One group was fertilized with fresh sperm as a control for egg quality, and the other group was fertilized with thawed sperm for fertility evaluation of cryopreserved samples. Fresh sperm were prepared by crushing of dissected testis in HBSS350 in an 80 times volume (l) of the testis weight (mg) yielding an concentration of 2…4 10^7 cells/ml. Fertility was recorded as the percentage of fertilized eggs observed at 6 h (early blastula stage) out of the total number of eggs, development of embryos was monitored daily, and hatching was recorded as the number of hatched “ngerlings at 14 d after fertilization divided by the total number of eggs.

Experiment I: Toxicity of six cryoprotectants on sperm motility without freezing

Evaluation of the effect of six cryoprotectants (methanol, ME, Me_2SO, DMA, DMF, and glycerol) on sperm motility was performed at “nal concentrations of 5%, 10%, and 15%. Sperm suspensions were prepared by pooling sperm from the testes of three males. Three replicates were prepared and measured for each concentration and incubation time. Sperm suspensions were mixed with double-strength cryoprotectant in HBSS350 at a “nal concentration of 2…4 10^7 cells/ml, and motility was monitored at 0, 10, 20, 30, 40, 50, and 60 min on ice.

Experiment II: Effect of cryoprotectants and cooling rates on sperm motility after thawing

Based on the evaluation of cryoprotectants on sperm motility in Experiment I, methanol, and ME (which showed the least reduction of sperm motility), and Me_2SO and DMF (used as cryoprotectants in the two previous publications) [1,15] at a “nal concentration of 10% were chosen for evaluation of the effect of cooling rate on post-thaw sperm motility. The sperm concentration at freezing was 2…4 10^7 cells/ml. In the “rst trial, sperm were frozen with three cooling rates (5, 19, and 23 C/min) with each cryoprotectant. In the second trial, tightened interval spacing was chosen for “ve cooling rates (5, 10, 15, 20, and 25 C/min) for each cryoprotectant except Me_2SO (which was dropped from consideration based on results in the “rst trial). Again, three replicates were produced with testes from “ve individuals pooled for each replicate.

Experiment III: Fertility testing of cryopreserved sperm with two freezing protocols

Based on the results of Experiment II, two combinations … methanol (10%) as cryoprotectant with a cooling rate of 10 C/min, and ME (10%) as cryoprotectant with a cooling rate of 15 C/min … were selected to cryopreserve sperm for fertility evaluation. In the “rst trial, sperm samples from 10 males were individually cryopreserved by each of the two combinations, and the “nal sperm concentration at freezing was 2…4 10^7 cells/ml. In the second trial, sperm samples from 20 males were individually cryopreserved with methanol (10%) and a cooling rate of 10 C/min only. After storage for 3 months, frozen samples were thawed for motility and fertility analysis as described above. Egg quality in the fertility test was evaluated by use of fresh sperm (control) collected from males of the same strain as described above.

Data analysis

Data were analyzed using SYSTAT 12 (Systat Software Inc., MI). Treatment effects were tested by using a two-sample t-test, analysis of variance (ANOVA), or repeated-measures ANOVA. The relationships among “sh characteristics, sperm motility and fertility after thawing were analyzed by SYSTAT correlations. Gonadosomatic index (GSI) was calculated as the percentage of testis weight divided by the body weight, and condition factor was calculated as the percentage of the body weight (g) divided by the cube of standard body length (cm). Percentage data were arcsine-square-root transformed for normalization before analysis. The signi“cance level was set at $P < 0.050$.

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Results

Biological characteristics

In total, the 74 males used in this study possessed a standard body length of 2.6 ± 0.1 cm (mean ± SD), body weight of 0.315 ± 0.050 g, and testis weight of 2.0 ± 0.6 (mg).

Experiment I: Toxicity of six cryoprotectants to sperm motility without freezing

The motility of fresh sperm before incubation with cryoprotectants was 84 ± 4% (mean ± SD). The capacity for sperm motility activation decreased with increased cryoprotectant concentration (5%, 10%, and 15%) and incubation time (5…60 min) for all six cryoprotectants (methanol, ME, Me $_2$SO, DMA, DMF, and glycerol) (Fig. 1). Overall, the motility of sperm varied signi“cantly depending on the cryoprotectants tested (P = 0.000), and the incubation time (P = 0.000) at all three test concentrations.

Glycerol reduced motility the most of the six cryoprotectants tested. The ability to activate motility of sperm decreased signi“cantly immediately after mixing in all three “nal concentrations (P 6 0.003). After incubation for 30 min, sperm motility activation decreased to <10% in all concentrations, and continued to decrease to 0% at 60 min with 5% and 10%, and at 40 min with 15%.

Dimethylsulfoxide, DMA and DMF were of intermediate toxicity. The ability to activate motility of sperm decreased signi“cantly immediately after mixing at “nal concentrations of 10% and 15% (P 6 0.009), but not at 5% (P = 1.000). After 50 min of incubation, motility at the 10% concentrations decreased to <10%, and after 30 min, motility at the 15% concentrations decreased to <10%. When a concentration of 5% was evaluated, motility decreased signi“cantly compared to the motility in control sperm (without cryoprotectants) after 30 min with Me $_2$SO (P 6 0.043), after 40 min with DMA (P 6 0.029), and after 10 min with DMF (P 6 0.029).

Methanol and ME were the least toxic of the cryoprotectants tested. The ability to activate motility at all three concentrations did not change after immediate mixing (P P 0.187). When methanol was used, the motility of sperm did not decrease compared with that of the control treatment until after 40 min with the 5% concentration (P 6 0.010), 30 min with the 10% concentration (P 6 0.012), and 10 min with 15% concentration (P 6 0.030). When ME was used, the motility of sperm after mixing was the same as that of the control after 40 min with concentrations of 5% and 10% (P P 0.062), and 10 min with a concentration of 15% (P P 0.073).

Experiment II: Post-thaw motility of sperm cryopreserved with different combinations of cryoprotectants and cooling rates

In the “rst trial, the cryoprotectants interacted with cooling rate. After cryopreservation with Me $_2$SO, methanol, ME, and DMF (10%) at three cooling rates (5, 19, and 23 C/min), the motility of sperm after thawing was signi“cantly decreased compared to that of fresh sperm (85%) before freezing (P 6 0.000), and the motility decrease was affected by cryoprotectant, cooling rate, and their interaction (ANOVA; P 6 0.000) (Fig. 2; upper panel). Analysis of the four cryoprotectants tested in the “rst trial showed that Me $_2$SO reduced the post-thaw motility signi“cantly compared to the other three cryoprotectants (P 6 0.000); the motility at all three cooling

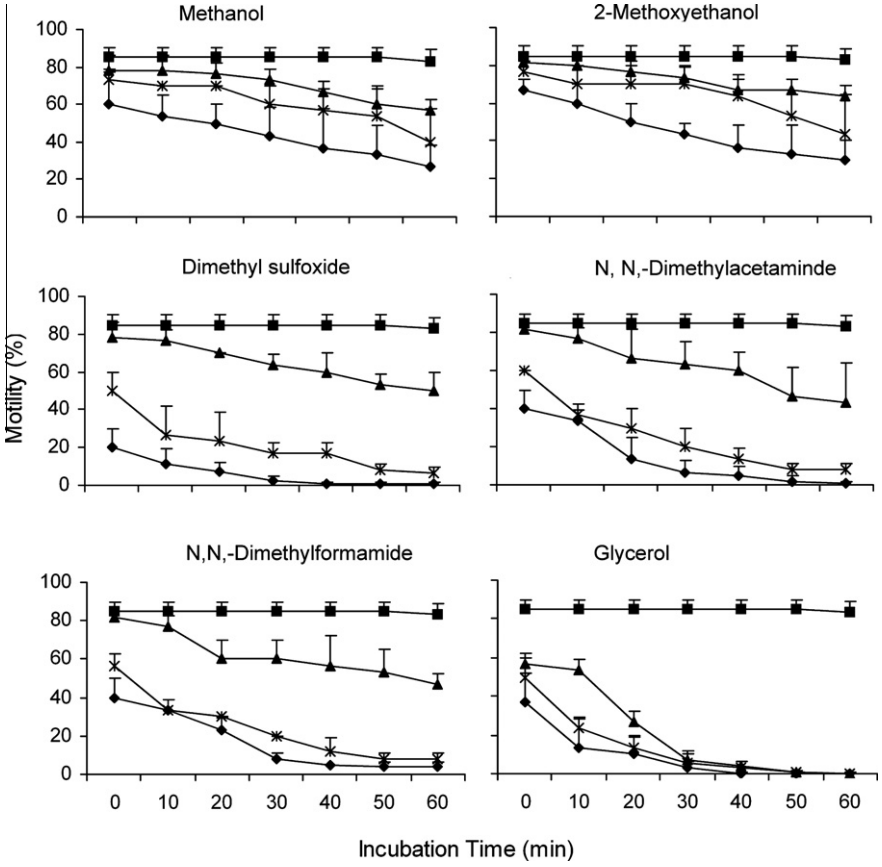

Fig. 1. The motility of sperm (mean ± SD, n = 3) of medaka Oryzias latipes after exposure to six cryoprotectants during 60 min at “nal concentrations of 0% (squares), 5% (triangles), 10% (stars), and 15% (diamonds) in Hanks• balanced salt solution at an osmolality of 350 mOsmol/kg on ice.

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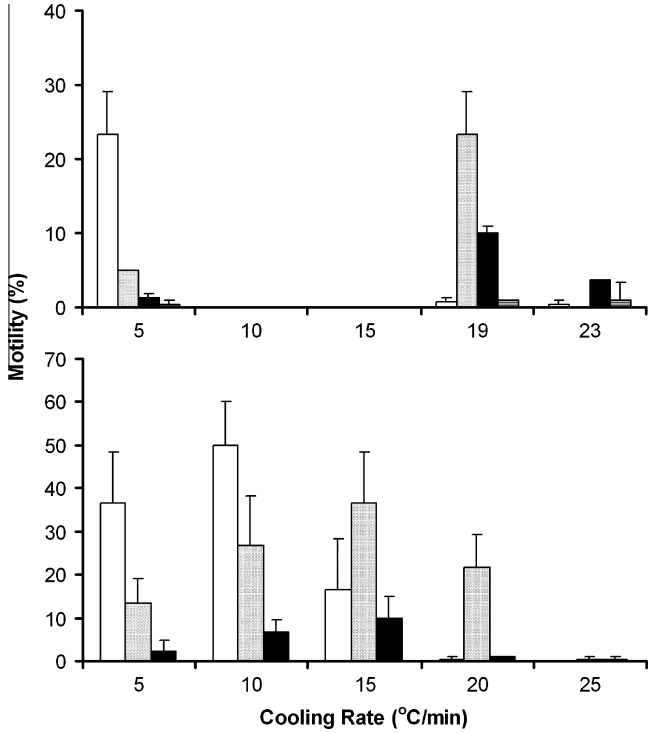

Fig. 2. Motility (mean ± SD) of thawed sperm with four cryoprotectants (at 10% concentration) at different cooling rates from 5 to 80 C in Trial 1(top panel), and Trial 2 (bottom panel): methanol, white; 2-methoxyethanol (ME), light gray; dimethyl sulfoxide (Me $_2$SO), dark gray (Trial 1 only), and N,N-dimethyl formamide (DMF), black. The motility of fresh sperm (before addition of cryoprotectants) was 85 ± 0% in Trial 1, and 83 ± 3% in Trial 2.

rates were all less than 1%, and were not different from one another (P P 0.358). When 10% methanol was used, motility at a rate of 5 C/min (23 ± 6%) was signi“cantly higher (P 6 0.000) than that for 19 C/min (1 ± 1%) and 23 C/min (0 ± 1%). When 10% ME was used, motility at 19 C/min (23 ± 6%) was signi“cantly higher (P 6 0.000) than that for 5 C/min (5 ± 0%) and 23 C/min (0 ± 0%). The same pattern was observed when DMF was used. A rate of 19 C/min yielded higher motility (10 ± 0%) than did 5 C/min (1 ± 1%) (P = 0.001) and 23 C/min (4 ± 2%) (P = 0.001).

In the second trial, a tighter spacing of cooling rates between 5 and 25 C/min was used to cryopreserve sperm with methanol, ME, and DMF as cryoprotectants (Me $_2$SO was not included because of the low post-thaw motility in the “rst trial), and the interaction of cryoprotectants and cooling rate was clearly evident. The post-thaw motility was signi“cantly affected by cryoprotectant (P = 0.000), cooling rate (P = 0.000), and their interaction (P = 0.000) (Fig. 2; lower panel) compared to that of fresh sperm (83 ± 3%) before freezing. When methanol (10%) was used as cryoprotectant, motility at a cooling rate of 10 C/min (50 ± 10%) was highest (P 6 0.003), and motility (37 ± 12%) did not change signi“cantly (P = 0.490) at 5 C/min. However, with the cooling rate increased to 15 C/min, motility declined signi“cantly to 17 ± 12% (P = 0.003), and to 0% when rate increased to 20 and 25 C/min (P 6 0.000). When ME was used, motility was not signi“cantly different at rates of 10 C/min (27 ± 12%), 15 C/min (37 ± 12%), and 20 C/min (22 ± 8%) (P 6 0.287). But, at rates of 5 and 25 C/min, motility was signi“cantly lower (P 6 0.027) compared to that at 15 C/min. When DMF was used, motility at all rates tested was 6 10%. The highest motility was observed at rates of 10 C/min (7 ± 3%) and 15 C/min (10 ± 5%).

Experiment III: Evaluation of methanol (10%) with a cooling rate of 10 C/min, and ME (10%) with a cooling rate of 15 C/min

In the “rst trial, no difference was detected (P = 0.546) in the motility of post-thaw sperm cryopreserved with methanol (10%) with a cooling rate of 10 C/min (50 ± 9%) and ME (10%) with a rate of 15 C/min (58 ± 11%) (Table 1). Also, no difference was observed in motility before freezing (P = 0.117). Of the “sh used for those two treatments, there were no differences in the body characteristics (including standard body length, body weight, and testis weight) (Table 1). However, during the process of in vitro fertilization in this trial, BSS buffer was added 2 h after the mixture of post-thaw sperm and eggs (rather than after the usual 1 min). For fresh sperm, this is not a necessary step; but for cryopreserved sperm, it meant that the gametes were exposed to the cryoprotectant (10% methanol or ME) for 2 h. The development at day 7 and hatch at day 14 from cryopreserved groups were all less than 10%. Based on these and our other studies, we concluded that these data were not representative of the actual fertility of cryopreserved sperm. In addition, we performed a separate study (data not shown) to evaluate the effect of 10% methanol and ME on in vitro fertilization by using fresh sperm. Abnormal embryos were again observed in the eggs fertilized with sperm after ME incubation for 10 min or longer before adding BSS. We suspect that prolonged exposure to this cryoprotectant exerted a teratogenic or mutagenic effect. This observation merits further investigation given reports of mutagenesis for this chemical [5] . This cryoprotectant was eliminated from further study.

In the second fertilization trial, the sperm concentration from each individual varied from 7 10^6 to 1 10^8 cells/ml (Table 2). The initial motility of fresh sperm (before freezing) from 20 males was 45 ± 22% ranging from 5% to 83%, and after thawing, the motility was 21 ± 10% ranging from 1% to 37% (Table 2) (note: the sample from “sh number 20 did not include sperm, but was processed for fertilization as part of the single-blind test). There was a significant correlation between initial motility of fresh sperm and the post-thaw motility (R^2 = 0.521) (P = 0.017) (Table 3). Thawed sperm showed an average fertilization of 82 ± 26% (from 10% to 100%), and an average hatching of 70 ± 30 (from 0% to 100%) (n = 19; excluding “sh number 20). Correlation analysis showed that fertilization and hatch produced by these thawed sperm were signi“cantly related to each other (R^2 = 0.664) (P = 0.001) (Table 3). Egg quality evaluation with fresh sperm showed an average fertilization of 97 ± 7% (from 70% to 100%), and an average hatching of

Table 1
Sperm motility (mean ± SD) of medaka Oryzias latipes (n = 10) before freezing and after thawing with two freezing treatments: methanol (10%) with a cooling rate of 10 C/min (Method 1), and 2-methoxyethanol (10%) with a cooling rate of 15 C/min (Method 2), and the associated male body characteristics. The P values are for comparisons made within columns (there were no signi“cant differences).

	Motility (%)		Body characteristics		
	Fresh	After thawing	Length (cm)	Weight (g)	Testis (mg)
Method 1	83 ± 9	50 ± 9	2.6 ± 0.1	0.309 ± 0.035	2.1 ± 0.4
Method 2	85 ± 7	58 ± 11	2.7 ± 0.1	0.349 ± 0.080	2.1 ± 0.4
P values	0.117	0.546	0.080	0.315	0.842