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A Procedure-Spanning Analysis of Plasma Membrane Integrity for Assessment of Cell Viability in Sperm Cryopreservation of Zebrafish *Danio rerio*

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Abstract

The goal of this study was to evaluate plasma membrane integrity and motility for zebrafish sperm quality assessment along the cryopreservation pathway—from sample collection through refrigerated storage, cryoprotectant equilibration, freezing, thawing, and fertilization. The objectives were to: (1) evaluate the effects of osmolality, extender, and refrigerated storage on sperm plasma membrane integrity and motility, and (2) compare cryopreservation of sperm from farm-raised and well-characterized research populations by evaluating motility and membrane integrity of fresh, post-equilibration (before freezing) and post-thaw sperm, and post-thaw fertility. Osmolality, extender, and storage time each influenced sperm motility and membrane integrity. Isotonic osmolality showed the best protection for motility and membrane integrity compared to hypotonic and hypertonic osmolalities. Of the four tested extenders, Hanks' balanced salt solution (HBSS) and Ca^{2+}-free HBSS showed the best protection compared with NaCl and glucose, and sperm retained motility and membrane integrity for 24 h of refrigerated storage. Sperm cryopreservation of zebrafish from a farm population ($n=20$) and an AB research line ($n=20$) showed significant differences in post-thaw fertility (32% ± 18% vs. 73% ± 21%). No differences were found in post-thaw motility, although the farm-raised zebrafish possessed a larger body size, testis weight, and higher fresh motility. Correlation analysis of pooled data did not identify correlations among motility, flow cytometry analysis of membrane integrity and recognizable cells, and post-thaw sperm fertility ($p \geq 0.202$). More research is needed to standardize the fertilization conditions especially sperm-to-egg ratio to avoid possible overabundance of sperm to obscure the differences.

Introduction

Cryopreservation is a useful and efficient technique for preservation of valuable research strains. Generally, the procedural pathways for cryopreservation activities involve initial development of cryopreservation protocols, application of the protocols, and large-scale application for genetic resources and commercial industry.[1] The initial pathway for developing cryopreservation protocols includes the following procedures: sperm collection, suspension of sperm in extender without motility activation, addition of cryoprotectants, sample packaging, cooling to cryogenic temperatures, and frozen sample storage.[1,2] Due to the connection of each step in this series of procedures, cell damage can occur at any step, is cumulative, and can result in final failure.[3] Therefore, it is necessary to develop effective, fast, and accurate methods for sperm cell quality assessment.

Zebrafish *Danio rerio* is one of the most widely used aquatic organisms for developmental, genetic, and biomedical research.[4] Sperm cryopreservation has been studied in zebrafish since the 1980s, and effective protocols have been developed and applied for recovery of mutant lines, long-term germplasm preservation, and artificial fertilization.[5–12] In these published protocols, the extenders for dilution of sperm samples included Ginsberg's buffer,[13] Hanks' balanced salt solution (HBSS),[5,14] and balanced Tris buffer (75 mM NaCl, 70 mM KCl, 2 mM CaCl2, 1 mM MgSO4, and 20 mM Tris) with or without addition of skim milk, fetal bovine serum, glucose, or other sugars. The cryoprotectants used include methanol, *N*,*N*-dimethyl acetamide (DMA), and *N*,*N*-dimethyl formamide (DMF) at concentrations of 8%–10%. Methodologies for cooling of samples have included programmable freezing systems with precise cooling rates, dry ice, and liquid nitrogen vapor.[15] Although these

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published protocols have achieved some success for sperm cryopreservation, improvement is still needed to reduce variation, and increase post-thaw sperm viability and fertility. In addition, due to the relatively small zebrafish body size (2–4 cm) and limited sperm availability (1–3 μL), there is a need for improvement and optimization of protocols to increase efficiency and post-thaw viability to make full use of cryopreserved sperm.[15]

To develop cryopreservation protocols, the choice of an extender solution is the first step. Extenders are usually buffers that are mixed with the sperm sample to maintain the capacity for sperm to become motile (i.e., prevent activation), protect the sperm cells from possible toxic byproducts, provide necessary nutrients, dilute the sample to a desired sperm concentration, and act as a carrier medium for the cryoprotectants needed during cryopreservation. Thus, formulation of ingredients, pH, temperature, osmolality, dilution, and additives (e.g., sugars or proteins) can all influence sperm viability.[16,17] For different fish species, sperm motility can be activated by changes in osmolality or ion conditions, or other factors depending on the mode of reproduction (i.e., viviparity, oviparity, or ovoviviparity) and habitats (i.e., freshwater, brackish water, or marine). Once motility is activated, sperm usually lose motility and fertility after a few minutes (e.g., most external fertilization fishes) or several days (e.g., viviparous fishes).[5,15–19]

For zebrafish, sperm are activated by hypotonic solutions and motility can persist for only a few minutes.[5,20] After mixing with extenders, sperm cells undergo osmotic exchange with the extenders resulting in changes to the plasma membrane (e.g., expansion or shrinkage). In addition, sperm suspended in extender can be chilled to reduce sperm metabolism and increase longevity for shipping or other applications, and thus refrigerated storage conditions can affect sperm viability. During cryopreservation, sperm cells are thought to face at least two major mechanisms of damage: solution effects caused by high concentration of solutes under suboptimal slow cooling, and intracellular ice formation, which occurs at high cooling rates.[21,22] Thus, cooling rate, extender type, cryoprotectant, and their interactions are all factors that exert an effect on plasma membrane integrity during the cryopreservation process.

Flow cytometry is a widely applied technique for analysis of cell suspensions including sperm samples, and it has been used for assessment of sperm quality by analysis of plasma membrane integrity, acrosome integrity, mitochondrial integrity, specific surface molecules (e.g., antigens), DNA breakage,[23] and apoptosis.[24] During cryopreservation, plasma membrane integrity is therefore a key factor to assess overall sperm quality, and flow cytometry is the most accurate and rapid method for assessing this parameter by using methods such as the membrane-permeant nucleic acid stain SYBR®-14 or the intercalating nucleic acid stain propidium iodide (PI).[25]

The goal of this study was to evaluate sperm plasma membrane integrity and motility for sperm quality assessment along the entire pathway from sample collection through refrigerated storage, freezing, thawing, and use in zebrafish from farm-raised and research populations. The objectives were to: (1) evaluate the effects of osmolality, extender, and refrigerated storage on sperm plasma membrane integrity and motility, and (2) compare cryopreservation of sperm from farm-raised and research populations by evaluating motility and membrane integrity of fresh, post-equilibration (before freezing), and post-thaw sperm, and post-thaw fertility.

Materials and Methods

Zebrafish

Male zebrafish, of optimal breeding age (between ~6 and 12 months old), were obtained from two sources: (1) the AB reference strain (wild-type) from the Zebrafish International Resource Center (ZIRC, Eugene, OR; zebrafish.org), and (2) uncharacterized *Danio* zebrafish from Segrest Farm (Gibsonton, FL, www.segrestfarms.com). These adults were maintained at a density of two fish/L at 26°C in a recirculating water system for zebrafish (Aquatic Habitats, Apopka, FL) at the Aquaculture Research Station of the Louisiana State University Agricultural Center. Photoperiod was set at 14 h light: 10 h dark and fish were fed twice daily with commercial pellets (Aquatic Eco-system, Apopka, FL) and *Artemia* nauplii hatched from cysts (INVE Group, Grantsville, UT). The filter system was back-flushed weekly. Protocol approval was obtained from the Institutional Animal Care and Use Committees of Louisiana State University and University of Oregon.

Sperm collection

Sperm were collected by crushing of dissected testes. Fish were placed on crushed ice for 1 min, and then blotted dry. After measurement of standard body length (from the tip of the snout to the base of the caudal fin) and body weight (mg), testes were obtained by dissection under a dissection microscope (×10 magnification), and weighed after placement into 1.5-mL microcentrifuge tubes. Sperm suspensions were obtained by crushing testes in extender as specified in each experiment at an initial ratio of 1:40 (mg: μL).

Sperm concentrations of pooled samples were determined using a micro-spectrophotometer (NanoDrop®; Thermo Fisher Scientific, Wilmington, DE). The absorbance of a 2-μL aliquot of sperm suspension was measured at a wavelength of 400 nm in triplicate, and sperm concentration was calculated from the average absorbance using an equation previously determined in our laboratory.[26] Cell concentration was adjusted to 2×10^8 cells/mL by dilution with extenders and samples were filtered through 30-μm nylon mesh to remove any remaining tissues.

Motility estimation

To estimate motility, 1 μL of sperm suspension was placed on a glass slide, and 9 μL of deionized water (or different volumes based on the experiment) were added to activate sperm motility. Motility was observed at 200-× magnification using a dark-phase microscope (Optiphot 2, Nikon, Inc., Garden City, NY), and expressed as the percentage of sperm that moved actively in a forward direction in relation to the total number of cells.

Fluorescent staining and flow cytometry

Sperm membrane integrity was assessed using the LIVE/DEAD® SYBR-14/PI assay (Molecular Probes; Thermo Fisher Scientific, Eugene, OR). Sperm (500 μL at a concentration of

1×10^6 cells/mL) were stained with SYBR-14 at a final concentration of 100 nM and PI at a final concentration of 12 μM in 1.5-mL microcentrifuge tubes, and incubated for 10 min in the dark at room temperature before flow cytometry analysis. Samples were analyzed using an Accuri C6 Flow Cytometer® (BD Biosciences, San Jose, CA) equipped with 488-nm and 640-nm lasers. Before analysis, verification of flow cytometer performance was performed using fluorescent validation beads (Spherotech beads; BD Biosciences) to ensure that coefficient of variation values for the fluorescence detectors were <3.0% (based on full peak height).

An analysis sample (10 μL) was collected at a medium flow rate (35 μL per min). SYBR-14 was detected with a 530 ± 15 nm band-pass filter and PI was detected with a >670 nm long-pass filter. Sperm population gating to exclude debris was based on forward scatter (FSC) versus side scatter (SSC) plots (CFlow® software version 1.0.202.1; BD Biosciences). These gated events were sperm cells that retained cell structure and were stained with SYBR-14 or PI, and were defined as ''Recognizable Cells'' in this study. Furthermore, the gated cells were viewed on a scatter plot showing FL1 (SYBR-14) versus FL3 (PI) with fluorescence compensation (spectral overlap of SYBR-14 in FL3 was compensated by 0.1%, and spectral overlap of PI in FL1 was compensated by 1.9%) calculated using the CFlow software following the manufacturer's instructions. Sperm stained with SYBR-14 were separated from the cells stained with PI, and were named as ''Membrane-intact Cells.'' In this study, the percentage of ''recognized cells'' was calculated to reflect sperm cells that retained cell structures (i.e., did not break into pieces) after each treatment. Membrane integrity was calculated as the percentage of membrane intact cells (stained with SYBR-14 only) out of the recognized cells (stained with SYBR-14 or PI).

Cryopreservation of sperm samples

For sperm cryopreservation, samples were mixed with an equal volume of 16% methanol in Ca^{2+}-free HBSS yielding a final concentration of 8% methanol and 1×10^8 cells/mL, equilibrated for 10 min on ice after packaging in 0.25-mL French straws, cooled at a rate of 10°C/min from 4 to −80°C in a programmable freezer (Kryo 10 series II; Planer Products, Sunbury-on-Thames, UK), and plunged into liquid nitrogen for storage in canisters in a dewar.[5]

Experiment I. Effect of osmolality, extender, and refrigerated storage time on sperm motility and membrane integrity

After dissection, testes pooled from five males were minced together on ice in a Petri dish, and separated into four groups evenly. Each part of the testes was suspended in one of the four extenders—HBSS (0.137 M NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.55 mM glucose, pH = 7.2), Ca^{2+}-free HBSS (i.e., HBSS without $CaCl_2$), NaCl, and glucose—at an osmolality of 300 mOsmol/kg in a 1.5-mL tube at a concentration of 2×10^8 cells/mL. The final osmolality was measured with a vapor pressure osmometer (Model 5520; Wescor, Inc., Logan, UT), and pH was determined with a pH meter (AB15; Thermo Fisher Scientific). For each extender, sperm samples were separated into four aliquots and mixed with equal volumes of deionized H_2O, or extenders at 300, 600, 1200, and 2400 mOsmol/kg to yield final osmolalities of 150, 300, 600, and 1200 mOsmol/kg. Shortly after mixing with the extenders (within 30 s and after 2, 24, 48, and 72 h of refrigerated storage at 4°C, sperm motility was observed by using a microscope at a ×200 magnification. Sperm were activated by dilution with deionized H_2O at the following ratios: 1:1.5 for 150 mOsmol/kg; 1:4 for 300 mOsmol/kg; 1:9 for 600 mOsmol/kg; and 1:19 for 1200 mOsmol/kg to ensure the final osmolality was 50–80 mOsmol/kg to activate sperm motility.[5] At the same time, sperm samples in each buffer at each osmolality were analyzed by flow cytometry. Three replicates were produced by pooling testes from five males for each replicate.

Experiment II. Assessment of motility, membrane integrity, and fertility of sperm samples by using zebrafish from farm-raised and ZIRC reference strain

Males from the commercial farm ($n = 20$) and ZIRC ($n = 20$) were used for sperm collection individually. Motility, membrane integrity, and frequency of recognizable cells were measured for fresh and post-thaw sperm. Functionality of the post-thaw sperm was tested by *in vitro* fertilization.

For fertilization, zebrafish eggs were collected at ZIRC by stripping. The females were anesthetized in 0.01% MS-222, rinsed in fish water (water from the fish tank), blotted dry on a paper towel (excess water will cause swelling of the eggs and prevent fertilization), and placed sideways in a sterilized 60 × 15 mm Petri dish. Eggs were released by gentle compression along the belly with damp fingers, and returned to fish tank water for recovery. Good quality eggs were used in this study,[9] and fresh control sperm from donor males was used to evaluate egg quality during fertilization trials. Within 5 min after collection, eggs from one or two females were pooled and separated into two groups (>75 eggs for each group) for fertilization with fresh control sperm collected by stripping from several males in HBSS300 or thawed sperm.

Before fertilization, frozen sperm samples in 0.25-mL French straws were thawed in a water bath at 40°C for 5 s, and the straw ends were clipped to release the sperm suspension onto the eggs in the Petri dish. Fish water with a volume of seven times that of the sperm suspension was added to activate the gametes; the time was recorded as the fertilization time. Five minutes after activation, embryo medium was added to fill the dish half way. At 30 min, the fertilized eggs were transferred to larger Petri dishes in fresh 0.5× E2 embryo medium (7.5 mM NaCl, 0.25 mM KCl, 0.5 mM $MgSO_4$, 75 μM KH_2PO_4, 25 μM Na_2HPO_4, 0.5 mM $CaCl_2$, and 0.35 mM $NaHCO_3$, 0.5 mg/L methylene blue, pH 7.2–7.6; www.zebrafish.org/documents/protocols/pdf/Fish_Nursery/E2_solution.pdf) and placed in a 28°C incubator. The fertilization rate was determined as the percentage of developing (blastula stage) embryos divided by the total number of eggs, and the reported data were normalized by considering the fertilization in control group as 100%. Observations were made at late blastula period (high stage) at 3–4 h after fertilization, and at approximately prim-5 (early pharyngula) stage[27] around 24 h after fertilization.

Data analysis

Sperm concentration data were normalized by logarithmic transformation, and percentage data were normalized by arcsine-square-root transformation before statistical analysis

using SYSTAT 13 (Ver. 13.00.05; SYSTAT Software, Inc., Chicago, IL). Results were considered significantly different at $p < 0.050$. Data were presented as mean ± standard deviation.

Results

The effects of osmolality, extender, and refrigerated storage time on sperm motility

Motility estimation showed that extender, osmolality, refrigerated storage time ($p < 0.001$), and their interactions ($p < 0.001$), all produced significant effects on sperm motility (Table 1). With the exception of HBSS and Ca^{2+}-free HBSS at osmolalities of 300 and 600 mOsmol/kg ($p \geq 0.218$), sperm motility decreased significantly after 2 h of storage ($p \leq 0.046$). At 24 h, sperm motility in all of the tested extenders had declined significantly ($p \leq 0.046$), and reached 0% except sperm stored in 300 mOsmol/kg HBSS, Ca^{2+}-free HBSS, and NaCl, whose motility continued to decline at 48 h and reached 0% at 72 h (Table 1).

Sperm suspended at 150 mOsmol/kg were activated in all four buffer types (motility: HBSS, 57% ± 8%; Ca^{2+}-free HBSS, 57% ± 8%; Glucose, 27% ± 18%; NaCl, 53% ± 3%), while osmolalities of 300, 600, and 1200 mOsmol/kg in all four extenders did not activate sperm motility. Sperm suspended in hypertonic 1200 mOsmol/kg showed significant decline in motility compared with other osmolalities ($p \leq 0.008$), and was 0% at 2 h (Table 1). Sperm suspensions at 1200 mOsmol/kg became a sticky mucous-like solution and only few sperm were observed. Although motility of sperm suspended at 600 mOsmol/kg did not show differences with that at 300 mOsmol/kg until 2 h ($p \geq 0.263$), sperm motility was 0% at 24 h for all of the four extenders, and gradually sperm suspensions at 600 mOsmol/kg also became mucous-like (Table 1). Sperm cells at osmolalities of 600 and 1200 mOsmol/kg were also observed to break into pieces after adding deionized H_2O for motility activation while viewing with a microscope (200× magnification). Of the four osmolalities, isotonic 300 mOsmol/kg retained the motility longest, especially in HBSS and Ca^{2+}-free HBSS (to 48 h).

Of the four extenders tested, glucose (without ions) performed the worst in maintaining sperm motility. Motility of sperm diluted in 300 mOsmol/kg glucose was significantly lower than other extenders assessed within 30 s after mixing ($p \leq 0.032$). NaCl (with ions but no buffering system) did not cause differences in sperm motility at 300 mOsmol/kg ($p \geq 0.159$) compared to that in HBSS and Ca^{2+}-free HBSS assessed within 30 s and at 2 h, but it did show a decline in motility after 24 h ($p \leq 0.004$). Motility of sperm was retained longest in HBSS and Ca^{2+}-free HBSS, and no differences in sperm motility were found between these two solutions at each osmolality and storage time ($p \geq 0.997$).

Effect of osmolality, extender, and refrigerated storage time on plasma membrane integrity

Overall, osmolality, extender, and refrigerated storage time significantly affected the percentage of recognizable cells ($p \leq 0.021$) and membrane integrity ($p \leq 0.001$; Table 2).

Sperm at isotonic osmolality of 300 mOsmol/kg showed the highest percentage of recognizable cells and membrane integrity for all four extenders. Compared to the samples in 300 mOsmol/kg, sperm samples at 600 mOsmol/kg did not show differences in the percentage of recognizable cells for storage time during 24 h ($p \geq 0.051$) and in membrane integrity for all storage times tested ($p \geq 0.053$). Sperm at 1200 mOsmol/kg showed a significant decrease in the percentage of recognizable cells ($p \leq 0.001$) and membrane integrity ($p \leq 0.020$) for all extenders after 30 min of storage. Sperm in 150 mOsmol/kg also showed a significant decrease in the percentage of recognizable cells ($p \leq 0.014$) and in membrane integrity ($p \leq 0.001$) for all four extenders after 30 min of storage.

The percentage of recognizable cells for sperm samples in the four extenders at all osmolalities did not change during 24 h of storage ($p \geq 0.051$). For membrane integrity, cells in HBSS, Ca^{2+}-free HBSS, and NaCl did not change for all osmolalities within 24 h ($p \geq 0.052$), but cells in glucose showed a significant decrease after 30 min of storage at 1200 mOsmol/kg osmolality ($p \leq 0.001$), after 2 h of storage

Table 1. Motility of Fresh Sperm (Percentage) of Zebrafish *Danio rerio* Suspended in Different Buffers at Different Osmolalities During Refrigerated Storage

Extender	*Osmolality (mOsmol/kg)*	*Within 30 s*	*2 h*	*24 h*	*48 h*	*72 h*
HBSS	150	70 ± 5	3 ± 2	0 ± 0	0 ± 0	0 ± 0
	300	68 ± 6	64 ± 1	37 ± 5	9 ± 4	0 ± 0
	600	65 ± 10	50 ± 21	0 ± 0	0 ± 0	0 ± 0
	1200	29 ± 19	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Ca^{2+}-free HBSS	150	68 ± 6	8 ± 5	0 ± 0	0 ± 0	0 ± 0
	300	68 ± 6	68 ± 6	37 ± 2	10 ± 5	0 ± 0
	600	65 ± 10	61 ± 15	0 ± 0	0 ± 0	0 ± 0
	1200	24 ± 11	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Glucose	150	34 ± 19	1 ± 2	0 ± 0	0 ± 0	0 ± 0
	300	42 ± 15	14 ± 11	0 ± 0	0 ± 0	0 ± 0
	600	38 ± 18	4 ± 3	0 ± 0	0 ± 0	0 ± 0
	1200	26 ± 16	0 ± 0	0 ± 0	0 ± 0	0 ± 0
NaCl	150	68 ± 6	2 ± 1	0 ± 0	0 ± 0	0 ± 0
	300	68 ± 6	43 ± 11	7 ± 7	0 ± 0	0 ± 0
	600	58 ± 15	30 ± 7	0 ± 0	0 ± 0	0 ± 0
	1200	23 ± 14	0 ± 0	0 ± 0	0 ± 0	0 ± 0

HBSS, Hanks' balanced salt solution.

Table 2. Flow Cytometry Analysis of the Percentage of Recognizable Cells and Percentage of Membrane-Intact Cells in Four Extender Solutions at Different Osmolalities During Refrigerated Storage for Fresh Sperm in Zebrafish *D. rerio*

Extender solution	Osmolality (mOsmol/kg)	30 min		2 h		24 h		48 h		72 h	
		Recognizable cells	Membrane integrity	Recognizable cells	Membrane integrity	Recognizable cells	Membrane integrity	Recognizable cells	Membrane integrity	Recognizable cells	Membrane integrity
HBSS	150	63 ± 10	68 ± 2	68 ± 25	82 ± 4	24 ± 11	71 ± 9	13 ± 1	43 ± 19	5 ± 2	18 ± 11
	300	100 ± 0	91 ± 2	90 ± 9	90 ± 1	82 ± 5	85 ± 3	76 ± 6	75 ± 4	53 ± 15	66 ± 5
	600	90 ± 7	93 ± 1	90 ± 10	91 ± 2	59 ± 29	88 ± 2	35 ± 16	82 ± 7	21 ± 14	75 ± 7
	1200	36 ± 7	97 ± 1	31 ± 8	98 ± 0	12 ± 15	98 ± 0	7 ± 4	97 ± 1	2 ± 3	96 ± 5
Ca^{2+}-free HBSS	150	37 ± 13	73 ± 6	32 ± 18	93 ± 3	21 ± 14	87 ± 11	2 ± 2	48 ± 19	0 ± 0	27 ± 23
	300	100 ± 0	91 ± 2	87 ± 5	92 ± 1	65 ± 19	87 ± 2	37 ± 18	51 ± 14	32 ± 31	18 ± 7
	600	90 ± 8	93 ± 0	81 ± 20	92 ± 1	37 ± 25	54 ± 31	22 ± 16	25 ± 32	16 ± 12	17 ± 27
	1200	38 ± 12	97 ± 1	11 ± 4	98 ± 0	1 ± 1	69 ± 29	0 ± 0	47 ± 17	0 ± 0	17 ± 21
Glucose	150	57 ± 36	69 ± 5	49 ± 49	87 ± 1	16 ± 14	35 ± 18	1 ± 1	10 ± 12	1 ± 0	9 ± 6
	300	100 ± 0	93 ± 2	93 ± 10	90 ± 5	38 ± 18	28 ± 35	13 ± 4	9 ± 14	9 ± 12	2 ± 2
	600	73 ± 7	75 ± 17	68 ± 17	57 ± 25	11 ± 9	6 ± 8	13 ± 11	4 ± 3	4 ± 4	4 ± 3
	1200	53 ± 13	49 ± 21	58 ± 31	32 ± 10	10 ± 5	3 ± 3	2 ± 2	7 ± 2	2 ± 1	12 ± 1
NaCl	150	53 ± 10	58 ± 11	26 ± 2	86 ± 3	12 ± 8	67 ± 25	3 ± 3	40 ± 31	1 ± 1	47 ± 48
	300	100 ± 0	88 ± 2	97 ± 5	89 ± 2	70 ± 6	77 ± 6	63 ± 17	70 ± 8	55 ± 10	67 ± 8
	600	87 ± 17	92 ± 0	93 ± 7	92 ± 0	42 ± 19	87 ± 2	36 ± 17	82 ± 5	28 ± 15	78 ± 4
	1200	36 ± 10	97 ± 1	20 ± 17	98 ± 1	9 ± 5	99 ± 0	6 ± 4	98 ± 1	4 ± 4	77 ± 34

at 600 mOsmol/kg ($p \leq 0.020$), and after 24 h storage at 300 mOsmol/kg ($p \leq 0.043$).

Cryopreservation of sperm from farm-raised and reference strain populations

Male fish from the commercial farmed population were significantly larger and heavier than those from the ZIRC research stock (see p values in Table 3), and consequently the testis weight was also heavier ($p < 0.001$). For motility estimation, although a difference was found in fresh sperm between the two populations, no significant difference existed in post-thaw motility ($p = 0.489$). The percentage of recognizable cells of fresh sperm in the farm-raised population was higher than that in the research stock ($p < 0.001$), but in post-thaw samples the opposite results were found ($p \leq 0.017$). Membrane integrity did not show differences between these two populations for fresh sperm ($p = 0.188$) or post-thaw sperm ($p = 0.060$) but did show a difference after equilibration with cryoprotectant before freezing ($p < 0.001$; Table 3). Fertility of the post-thaw sperm from research stock at 4 h (62% ± 14%) and 24 h (73% ± 21%) was significantly higher than that in the farmed population at 4 h (27% ± 15%) and at 24 h (32% ± 18%) ($p < 0.001$). The increase of post-thaw survival at 24 h over fertilization at 4 h was from the data normalization, which considered the fertilization in control group as 100%.

Correlation analysis for the farmed 20 males showed that body length, weight, and testis weight were significantly correlated with each other ($p \leq 0.033$), also correlations were found between post-thaw motility and membrane integrity ($p < 0.001$), and between fertilization at 4 h and survival at 24 h ($p < 0.001$). Other measurements did not show correlation to each other ($p \geq 0.068$). For the 20 males in the reference AB strain, correlations were found between body length and body weight ($p = 0.001$), membrane integrity of fresh sperm and that after equilibration ($p < 0.001$), recognizable cell and membrane integrity of post-thawing ($p < 0.001$), and fertilization at 4 h and survival at 24 h ($p < 0.001$); however, no correlations were found among other measurements ($p \geq 0.087$).

By pooling all of the measurement for the 40 males from both populations together, correlation analysis showed that body length, body weight, and testis weight obtained from these fish were always significantly correlated with each other ($p \leq 0.000$), and that these three biological measures were also correlated with fresh sperm motility ($p \leq 0.025$). However, fresh sperm motility and post-thaw motility were not correlated with each other ($p = 1.000$), and they did not correlate with the membrane integrity measured in fresh sperm, postequilibrium sperm, or post-thaw sperm, or in fertility tested at 4 h and survival at 24 h after fertilization ($p \geq 0.876$). In addition, the fertility of post-thaw sperm did not show correlations with the percentage of recognizable cells or membrane integrity ($p \geq 0.202$).

Discussion

This study investigated the utility of flow cytometry to measure plasma membrane integrity and percentage of recognizable cells and sperm motility for sperm quality assessment across the steps of the cryopreservation procedure, including refrigerated storage of sperm in different extenders

Table 3. Sperm Cryopreservation for Zebrafish *D. rerio* from a Farm-Raised Population ($N=20$) and the AB Reference Line ($N=20$)

Source	Value	Biological factors			Motility (%)		Post-thaw fertility (%)		Recognizable cells (%)		Membrane integrity (%)		
		Length (mm)	Weight (g)	Testis (mg)	Fresh	Post thaw	At 4 h	At 24 h	Post equilibration	Post thaw	Fresh	Post equilibration	Post thaw
Farm-raised $N=20$	Minimum	26.66	0.363	2.7	60	7	4	4	87	30	84	77	35
	Maximum	32.58	0.571	9.7	80	50	58	65	100	100	95	91	66
	Mean	30.06	0.511	7.2	68	25	27	32	95	82	92	86	48
	SD	1.40	0.057	1.8	8	16	15	18	5	16	3	4	8
Research AB Line $N=20$	Minimum	22.18	0.212	2.2	70	10	36	36	83	45	80	88	38
	Maximum	28.19	0.418	6.2	85	55	83	100	95	88	94	94	53
	Mean	25.02	0.312	3.6	80	28	62	73	89	74	91	92	45
	SD	1.25	0.053	1.0	6	15	14	21	3	9	4	1	4
p		0.000	0.000	0.000	0.000	0.489	0.000	0.000	0.000	0.017	0.188	0.000	0.060

Body length, body weight, testis weight, fresh motility, post-thaw motility, percentage of recognizable cells, and membrane integrity by flow cytometry analysis in fresh, post-equilibration and post-thaw sperm, and post-thaw fertility checked at 4 h and survival at 24 h after *in vitro* fertilization.
SD, standard deviation.

and different osmolalities, post-equilibration, and post-thawing in farmed and research populations of zebrafish. The results indicated that flow cytometry analyses reflected the effects of osmolality, extender, and storage time on sperm viability during refrigerated storage. Although differences in flow cytometry analyses, motility, and fertility were useful to evaluate the sperm viability between the farmed and research populations, flow cytometry analyses and motility did not show consistent correlation with the post-thaw sperm fertility under the fertilization conditions used.

Osmolality, extender, and storage time on fresh sperm motility

Identification of suitable extenders is essential for sperm dilution, storage, and cryopreservation. The function of an extender solution is to maintain the capacity for sperm motility by preventing activation of sperm motility. For most fishes, sperm are quiescent in the testes, and become motile after release into the environment.[15] Motility initiation and swimming duration are usually related to the reproductive modes and habitats, and thus osmolality, ionic concentrations, temperature, and pH can be factors that control or influence sperm motility initiation.[16,17]

For freshwater and marine species, motility initiation is controlled by osmolality (hypotonic for freshwater species such as zebrafish[5,20] and hypertonic for marine species[28]) and sperm can remain motile after activation for a few minutes. For euryhaline fishes, such as medaka,[29] *Fundulus*,[30] and tilapia,[31] osmolality and ions (e.g., calcium) are factors that can initiate sperm motility, and sperm can remain motile for minutes or hours. For diadromous fish, motility activation can rely on the concentration of ions such as potassium, for example in anadromous salmonid fishes,[32] and in species such as herring (*Clupea pallasii*) motility is activated by a protein that controls calcium influx and sodium efflux.[33] For viviparous fish, sperm motility can be initiated at isotonic osmolalities such as for *Xiphophorus* fishes,[18] or by a combination of osmolality and calcium ions, such as for *Xenotoca eiseni* from the Goodeidae family (our unpublished data). Because the natural habitat of zebrafish ranges between diluted (by rainwater; in the range of 11–22 μS/cm conductivity) and regular freshwater bodies such as rice paddies, ponds, small and slow-moving rivulets and streams to brackish water,[34,35] all these factors are crucial considerations when choosing extenders for zebrafish sperm storage and cryopreservation.

To date, extenders reported for sperm storage and cryopreservation have included many different buffers sometimes with additives including milk, egg yolk, proteins, and sugars,[1,36] and no standards exist. In this study, we intended to assess membrane integrity as an assay for sperm viability during refrigerated storage. Therefore, we chose glucose (without ions), NaCl (providing ions but no buffer system), HBSS (a buffer system), and Ca^{2+}-free HBSS (a buffer system with ions excluding calcium). The results of motility evaluation indicated that an ionic buffer system (HBSS, Ca^{2+}-free HBSS) protected sperm cells better than did glucose or NaCl alone, and that calcium did not make a significant difference. Osmolality played an important role in refrigerated storage, and sperm stored in isotonic extenders (300 mOsmol/kg) had the highest motility and longest storage time (as long as 48 h).

Analysis of membrane integrity and recognizable cells by flow cytometry also reflected the sperm viability at different conditions during storage. Due to the selective permeability of the sperm plasma membrane,[37] extenders with different osmolalities (solute concentrations) and buffer types (ions and molecular weights) can interact with the intracellular isotonic cytoplasm (usually $\sim$290 mOsmol/kg), and influence sperm viability during refrigerated storage and cryopreservation.[36] In this study, significant losses of recognizable cells and membrane integrity were identified in sperm samples suspended in 600 and 1200 mOsmol/kg. In addition, a mucus-like consistency was observed due to the possible damage of sperm cells by hypertonic solutions. A significant decrease in membrane integrity of sperm cells was observed when stored in glucose compared to storage in HBSS, Ca^{2+}-free HBSS, and NaCl, although no differences in the percentage of recognizable cells were found within a 2-h storage time.

Overall, osmolality, ionic composition (and additives), and storage time are all important factors to consider when

choosing extenders. Assessment of sperm motility, membrane integrity, and recognizable cells by using flow cytometry can be used to evaluate sperm viability at different storage conditions.

Correlation of motility, membrane integrity, recognizable cells, and post-thaw fertility

To test whether motility, membrane integrity, and percentage of recognizable cells were correlated with post-thaw sperm viability and fertility, zebrafish from farm and research populations were cryopreserved and compared. Although differences were found between the two tested populations in body sizes, testis weight, fresh sperm motility, post-thaw sperm motility, percentages of recognizable cells, and membrane integrity at the postequilibration and post-thaw stages, the only correlations identified among the measured indexes were among body size, testis weight, and fresh sperm motility. Furthermore, measurements of sperm membrane integrity in these two populations were not correlated with post-thaw fertility. For example, the farm-raised population had larger body sizes and testes, and higher fresh motility, but had no differences in post-thaw motility, and had lower post-thaw fertility than fish from the reference population.

The insensitivity of sperm quality assessment by using flow cytometry or motility in predicting post-thaw fertility in this study could be due to differences in the response to cryopreservation by the zebrafish from these two populations, or be due to the conditions used for fertilization. The significant difference in postequilibration membrane integrity between the farm-raised and research populations suggests that there was a difference in the response to cryoprotectants between the two populations, but this difference was not reflected in the post-thaw membrane integrity or fertilization data. The AB-strain has been propagated for approximately four decades with natural crosses through *in vitro* fertilization. Several times during its history in captivity, genetic diversity improved by using cryopreserved stocks from previous generations.[13,38] In contrast, farm-raised fish have never experienced such selection events in the farm environment. Similarly, mouse,[39] turkey,[40] and rooster,[41] animals from different lines showed inconsistent and even completely different post-thaw sperm viabilities although the same cryopreservation protocols were used. In this study, data analysis was based on 40 total males from the two populations, and we cannot exclude the possibility that more individuals and a tighter control of fish age between the two populations are needed for further analysis.

In addition, more research is needed to standardize and optimize fertilization conditions. An example would be inclusion of volumetric control of sperm during fertilization for control of sperm-to-egg ratios. At present, possible overabundance of sperm relative to eggs numbers (>75 eggs in these experiments) could obscure the differences in membrane integrity and motility of the post-thaw sperm.

For almost all cryopreservation programs, fertilization has been considered the main criterion for evaluation of cryopreservation success. However, for most animal species, *in vitro* fertilization is time and resource consuming, and requires females for egg collection. Overall, the procedure of cryopreservation involves a series of interconnected steps. Development of effective, fast, and accurate sperm quality assays is highly important and needed for each step of the pathway for the cryopreservation process. This research is an early trial for this research topic, and more investigation is needed.

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